# CYTOKINE GENE POLYMORPHISM ANALYSIS AND HPV TYPING IN LOW GRADE CERVICAL LESIONS

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**ABSTRACT**

Persistent high-risk human papillomavirus infection (HPVI) of the uterine cervix is associated with squamous intraepithelial lesions (SIL) and cervical cancer. Low-grade SIL (LSIL) is common but factors determining the outcome are incompletely understood and the management is controversial. The infecting HPV type and the host response to HPVI are thought to be important.

Genetic polymorphisms in the regulatory sequences of genes have been identified that are associated with different levels of cytokine expression. These include interleukin 1 alpha (IL-1α, 46 base pair VNTR in intron 6), interleukin 1 receptor antagonist (IL-1Ra, 86 bp VNTR in intron 2) and tumour necrosis factor alpha (TNFα, single nucleotide polymorphisms at –308 and -238).

Cytokine gene polymorphisms and HPV types were investigated in 209 women presenting to colposcopy clinic with smears suggesting LSIL and compared to controls with normal cervix and those with high-grade SIL (HSIL). Twelve month follow up showed 9% progression to HSIL, 15% persistence of LSIL, 56% regression and 21% loss to follow up.

High risk HPVI was common (62%) in women with smears suggesting LSIL but more so in those with co-existing HSIL (85%). HPV 16 was the most common infecting type but 14 infecting high-risk HPV types were seen. The risks of progressing or regressing were not influenced by the presence of HR HPV in this study. Screening for HPV types 16 and 18 alone would miss 36% of disease progression.

The low secretor phenotype of TNFα -308 was associated with all grades of SIL but most strongly with LSIL (p=0.004). The low secretor phenotype of IL-1α was associated with LSIL (p=0.031) but not with HSIL. No associations were demonstrated between IL-1Ra polymorphism and cervical disease. No associations were shown between cytokine polymorphisms and HPVI or disease outcome.

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**List of Abbreviations**

| | |
|---|---|
| ASCUS | Abnormal Squamous Cells of Undetermined Significance |
| ALTS | ASCUS/LSIL Triage Study |
| Bp | Base Pair |
| BSCC | British Society for Clinical Cytology |
| CGIN | Cervical Glandular Intraepithelial Neoplasia |
| CIN | Cervical Intraepithelial Neoplasia |
| COCP | Combined Oral Contraceptive Pill |
| DNA | DeoxyriboNucleic Acid |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| EIA | Enzyme ImmunoAssay |
| FISH | Filter InSitu Hybridisation |
| GP | General Practitioner |
| HPV | Human Papilloma Virus |
| HPVI | Human Papilloma Virus Infection |
| HSIL | High-grade Squamous Intraepithelial Lesion |
| IHG | Induced Heteroduplex Generator |
| IL | InterLeukin |
| ISH | InSitu Hybridisation |
| IUCD | Intra Uterine Contraceptive Device |
| LBC | Liquid Based Cytology |
| LLETZ | Large Loop Excision of the Transformation Zone |
| LSIL | Low-grade Squamous Intraepithelial Lesion |
| MHC | Major Histocompatibility Complex |
| NHS | National Health Service |
| NHSCSP | National Health Service Cervical Screening Programme |
| NICE | National Institute for Clinical Excellence |
| OCP | Oral Contraceptive Pill |
| ORF | Open Reading Frames |
| PCR | Polymerase Chain Reaction |
| RNA | RiboNucleic Acid |
| SCJ | Squamocolumnar Junction |
| SDS | Sodium Dodecyl Sulphate |
| SIL | Squamous Intraepithelial Lesion |
| SNP | Single Nucleotide Polymorphism |
| TBE | Tris-boric acid-EDTA |
| TNF | Tumour Necrosis Factor |
| TZ | Transformation Zone |
| VNTR | Variable Number of Tandem Repeats |

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**Aims of this thesis**

To determine the incidence and type of HPV infection in women presenting to colposcopy clinic with smears suggestive of low-grade cervical disease and to correlate HPV type with initial histological diagnosis.

To compare the cytokine gene polymorphisms found in IL-1α, IL-1Ra and TNFα in women with normal cervical cytology and with histological low-grade and high-grade cervical disease.

To examine the disease outcome in women with low-grade cervical disease in relation to HPVI and cytokine gene polymorphism.

**Hypothesis**

Persistent high risk HPVI is strongly associated with both CIN and cervical cancer. Women with a reduced Th 1 (pro-inflammatory) cytokine response to HPV are likely to be susceptible to viral persistence and therefore, potentially more vulnerable to worse disease outcomes. Cytokine gene polymorphisms affecting levels of Th 1 cytokine production my affect the individual's response to HPVI and thereby the evolution or resolution of early cervical dysplasia.

**Publications and Awards related to this thesis**

Kirkpatrick A, Bidwell J, van den Brule AJC, Meijer CJLM, Pawade J, Glew S. 2004 TNFα polymorphism frequencies in HPV- associated cervical dysplasia *Gynecol Oncol* 2004 Feb;92(2):675-9
(see Appendix 3)

Kirkpatrick A. 2000 BSCCP Travelling Scholarship. To visit Prof Walboomers laboratory at Vrije Universiteit, Amsterdam

# INTRODUCTION

## 1.1 The Normal Cervix

### 1.1.1 Embryology

The cervix forms at the base of the uterus and the top of the vagina from the fusion of the Mullerian ducts and the vaginal plate. It comprises the ectocervix and the endocervix. The Mullerian epithelium gives rise to columnar epithelium and the vaginal plate gives rise to squamous epithelium. The columnar epithelium is in continuity cephalad with the endometrium and extends caudally to a junction with the squamous epithelium of the vagina at a fixed point at, or just inside the cervical os. This is known as the original squamocolumnar junction (SCJ). The squamous epithelium continues caudally to a junction with vulval epithelium.

The site of the original SCJ varies in relation to the cervical os depending on the age of the woman and the presence of oestrogenic stimulation. The prepubertal cervix is small and the original SCJ commonly lies just inside the external cervical os. In late fetal life (due to the effect of maternal oestrogen), at puberty, in pregnancy and during use of the combined oral contraceptive pill (COCP) the cervix is exposed to increased oestrogen levels. This causes the cervix to become more bulky and a process of eversion occurs where the columnar epithelium is visible outside the external cervical os. This process is reversible after birth, after delivery, after stopping the COCP and after the menopause. Oestrogen also effects the vagina by lowering the pH and producing a more acid environment. When the columnar epithelium is exposed to this acid environment it undergoes metaplastic transformation. The original SCJ hence forms the outer border of the area known as the transformation zone (TZ). Within this area, epithelium undergoes transformation or metaplasia from columnar to squamous. When this epithelium is visible to the naked eye it is called a cervical ectropion or erosion.

Following the menopause the effect of oestrogen is removed and the cervix undergoes inversion where the original SCJ retracts into the

endocervix. The site of the original SCJ varies in about four per cent of normal women and in those exposed to diestrol stilbestrol in fetal life. In these women the Mullerian columnar epithelium extends beyond the ectocervix and into the vaginal fornices, giving a much larger transformation zone (known as a congenital transformation zone) to undergo metaplasia.

### 1.1.2 Metaplasia

This describes the process by which columnar epithelium changes to squamous epithelium. In 1910, Meyer suggested that offshoots from the original squamous epithelium undermined and detached the columnar epithelium. More recently it is apparent that the process begins with the development of pluripotent reserve cells at the base of the columnar epithelium. They mature and replace the surface layer of columnar cells, which are lost in every day life. These cells have round nuclei and little cytoplasm. Metaplasia appears to be stimulated by changes in the vaginal pH. Acidic vaginal secretions cause destruction of the columnar epithelium and stimulate the production of reserve cells. These reserve cells mature and differentiate; the nuclei enlarge with prominent nucleoli and the amount of cytoplasm increases. Stratification is limited and the epithelium remains thin. As the process continues the cells become more differentiated, stratified and more like mature squamous epithelium. In some cases the surface epithelium is mature while the deeper layers remain somewhat undifferentiated. There may also be excessive surface maturation with keratinisation. It is also common to see irregularity of the epithelial stromal junction with stromal papillae (incursions of squamous epithelium into the stroma) giving the impression of invasive buds. These features also appear in congenital transformation zones.

### 1.1.3 Transformation zone

It is clear that the cervical epithelium in this area is undergoing almost continual change, which is more marked at particular times of life. It is this change which makes it susceptible to acquiring a neoplastic potential.

## 1.2 Cervical screening

### 1.2.1 History

Cervical screening is a method of preventing cancer by detecting and treating abnormal changes in a woman's cervix. The first stage in cervical screening is the smear test (Pap test). This involves using a spatula to sweep around the cervix and take a sample of cells, which are smeared onto a slide and sent to a laboratory for microscope examination (Papanicolaou 1954).

Cervical screening began in Britain in the mid 1960s. Although many women were having regular smears by the mid 1980s, there was concern that those at greatest risk were not being tested and those who had positive results were not being followed up and treated effectively. The incidence of cervical cancer in Great Britain was in the middle of the European range but the mortality was second only to that in Denmark (Reeves 1998). The NHS cervical screening programme was set up in 1988 when the Department of Health instructed all health authorities to introduce computerised call-recall systems and to meet predetermined quality standards. The computerised call-recall system also included follow up investigations and treatment.

The work for this thesis was undertaken between 1999 and 2001. Practice will be reviewed as it was then rather than how it is currently. At that stage the NHS Executive's policy was that women between 20 and 64 were invited to have a cervical smear test every three to five years. Around 60% of health authorities invited women every three years and 15% had a mixed policy inviting women every three or five years depending on their age. The remainder, including Avon, invited women every 5 years. Research at that time suggested the cumulative incidence of cervical cancer is reduced by screening 20-64 year olds five yearly by 83.6%, three yearly by 91.2% and annually by 93.3% (Hakama 1986).

The NHSCSP revised standards were published in April 2004 setting national guidelines (NHSCSP 2004). These standards called for the age of first screening to be deferred to 25 years old and continue three yearly until age 49 with five yearly screening from 50-64 years old. Women over this age are screened if they have had a recent abnormal test or if they

had not been screened after age 50. This was based on more recent research showing that five-yearly screening offers a reduction in cancer incidence of 83% at ages 55-69 years and annual screening offers an 87% reduction. Three-yearly screening offers additional protection (84%) over 5-yearly screening (73%) for cancers at ages 40-54 years, and is almost as good as annual screening (88%). In women aged 20-39 years, even annual screening is not as effective (76%) as 3-yearly screening in older women (Sasieni 2003).

### 1.2.2 Organisation

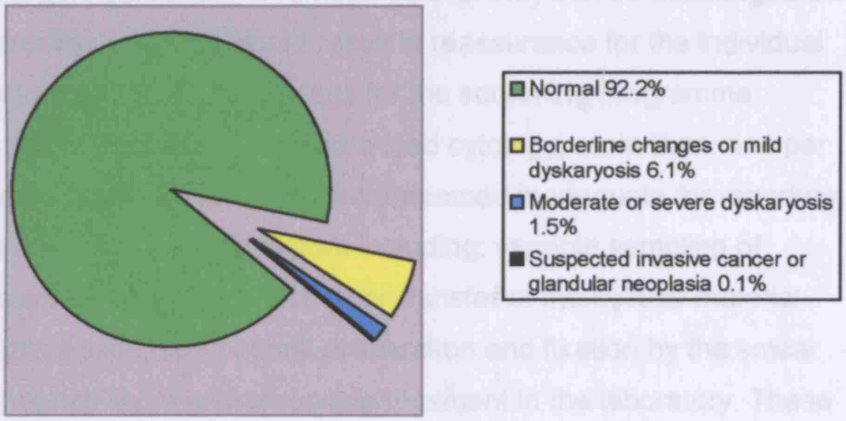
At the time of the work for this thesis the National Co-ordinating Office, based in Sheffield, was responsible for the overall performance of the programme. It was set up in 1994 with the aim to develop systems and guidelines which will assure a high quality of cervical screening throughout the country, identify important policy issues, help resolve them and improve communications within the programme and to women.

The programme screened almost four million women in England each year. In 1997/1998 2.3 million women were tested as a direct result of invitation and 1.6 million were screened opportunistically at the suggestion of the smear taker or the woman herself. Since some women had more than one smear in the course of a year, 4.4 million smears were examined by the pathology laboratories (DoH 1999). More recently in 2003-2004 3.6 million women were screened with 4 million smears examined (NHSCSP 2005). Under the GP contract of 1990, GP's received target payments for encouraging their patients to be screened. The basic target payment applied if 50% of eligible women were screened in the previous five years and a higher target payment if 80% were screened. Coverage hds almost doubled from 45% in 1988/1989 to 85% in 1997/1998 (Quinn 1999).

The cost of cervical screening, including the treatment of pre-cancerous lesions, is estimated at £132 million a year in England.

### 1.2.3 Results of the screening programme

*Figure 1.1* NHSCSP Smear Results in 1999




### 1.2.4 Effect

In 1997, 1,222 women died of cervical cancer, a death rate of 37 per million. The death rate increases with age, almost 95% of deaths occur in women over 35. Deaths from cervical cancer have fallen by nearly 50% from 71 per million in 1979 to 37 per million in 1997. According to the Imperial Cancer Research Fund, the NHS Cervical Screening Programme prevents between 1,100 and 3,900 cases of cervical cancer each year, nearly half all potential cases (Sasieni 1996). The fall in mortality, which began in the 1950s, accelerated in 1988 when the NHSCSP was set up, and mortality was initially falling at around 7% per year (Sasieni 1995). The NHSCSP exceeded the target set for it in "Health of the Nation" in 1986 of reducing the incidence of cervical cancer by at least 20% by the year 2000. This desired 80% level of coverage has been achieved since 1992. In 2004 there were 1093 deaths, a fall of 10.5% over 7 years since 1997 (Office of National Statistics 2005).

### 1.2.5 Drawbacks of the screening programme.

The reduction in cervical cancer mortality has also been achieved at the expense of poor specificity. Many more women than would ever have developed cervical cancer have been found to have an abnormality for

which they have been investigated and in some cases treated, when the underlying abnormality was harmless. The 1997 Guidelines for Clinical Practice and Programme Management of the NHSCSP (NHSCSP 1997) emphasise the need to define low risk women, whose chance of developing cervical cancer is remote, so that they can be discharged from further investigation. This would result in reassurance for the individual woman and significant cost savings for the screening programme.

In 1997, before the advent of liquid based cytology, more than nine per cent of smears sent for reporting were deemed inadequate for reporting. This was due to a variety of factors including: variable sampling of appropriate cells from the cervix, poor transfer of the cellular material onto the glass slide, sub-optimal preparation and fixation by the smear taker and variability of microscope assessment in the laboratory. These smears need to be repeated, causing distress and inconvenience to the woman involved and significant cost implications.

Liquid based cytology (LBC) has been shown to significantly reduce this problem of inadequate smears (Harkness 2003). The sample is collected in a similar way to the conventional smear but with a "broom like" spatula. The head of the spatula is rinsed in preservative fluid and this is sent to the laboratory where the cells in suspension are deposited onto a slide. Filters can be used to remove unwanted material such as red and white blood cells. There are two automated slide preparation systems, AutoCyte (formally CytoRich) and ThinPrep. It was thought that LBC may reduce the number of inadequate smears and possibly increase the productivity of screening laboratories by producing smears that are easier to read. LBC was introduced into the screening programme in several countries including the United States. The National Institute of Clinical Excellence (NICE) initially judged the evidence in favour of LBC was insufficient and of variable quality to justify nation-wide introduction of LBC (NICE 2000). However, following favourable pilot projects in the UK to investigate the effects, costs and practical implications of introducing LBC into the screening programme the phased introduction of LBC has been recommended (NICE 2003). By 2005 the national rate of inadequate smears had barely fallen to 9% (NHSCSP 2005). The rate of

inadequate smears at a Bristol LBC pilot site laboratory was 0.6% in the same year (local data). It should be noted that most inadequate smears occurred in under 25 year olds who are now not being screened and this may also contribute to reductions in the rate of inadequate smears (NHSCSP 2005).

### 1.2.6 Colposcopy

Colposcopy is used where suspicion of cervical disease exists, based either on cytology or on symptoms. It is a microscope providing illuminated magnification, which allows the cervix to be viewed at 6 to 40 fold magnification. The colposcope was developed by Hinselman in the 1920s and has gained popularity in the UK since the 1970s. It uses a tungsten or halogen light source with the option of adding a fibre optic cable. Most colposcopes have a focal length of 200-300mm allowing a comfortable working distance for the observer. The colposcope is binocular and may have the option of a side arm for an additional observer or attachment for a video or still camera.

### 1.2.7 Tissue basis for colposcopy

The colposcopic appearances are based on a number of factors;

1. The architecture of the epithelium and possible variations in its thickness and formation
2. The composition of the underlying stroma
3. The surface configuration of the tissue.

The image seen is based on the reciprocal relationship between these characteristics. The epithelium is colourless and acts as a filter through which the incident and reflected light passes. The stroma is coloured red by the blood vessels passing through it. The redness is reflected back to the observer but modified by the epithelium through which the light passes.

In the normal cervix during reproductive life, glycogen containing squamous epithelium is thick and multi-layered and acts as an effective filter, giving a pink to red appearance when viewed colposcopically. The columnar epithelium is thin, contains mucus and is highly translucent giving a dense red colposcopic appearance. Metaplastic epithelium at the transformation zone may be thinner than normal squamous epithelium

and devoid of glycogen giving an intermediate reddish pink appearance. Post menopausal (and prepubertal) epithelium is thinner with little glycogen and a reduced stromal blood supply, giving a pale red colposcopic appearance.

Abnormal epithelium (CIN) is more cellular with higher nuclear content that results in a more opaque appearance. Acetic acid causes coagulation of the nuclear protein. Normal cells still appear pink as the amount of nuclear protein is such that the vessels in the underlying stroma remain visible. Abnormal cells have a higher nuclear cytoplasm ratio and a higher nuclear protein density; this undergoes more coagulation and the cells appear white as the underlying vessels are obscured. The more abnormal the cell, the higher the concentration of protein and the whiter the cells appear.

When inflammatory infiltration occurs within the stroma the epithelial appearance may be altered to greyish white or yellow depending on the degree of infiltration.

The surface configuration is determined by the surface shape and by variations in epithelial thickness. Leukoplakia (thick keratin covering normal or abnormal epithelium) may appear as white patches. Vascular patterns may be visible; capillaries shine through the epithelium giving a punctate appearance or may run within stromal papillary ridges forming discrete fields (mosaicism).

*Figure 1.2* Mosaicism

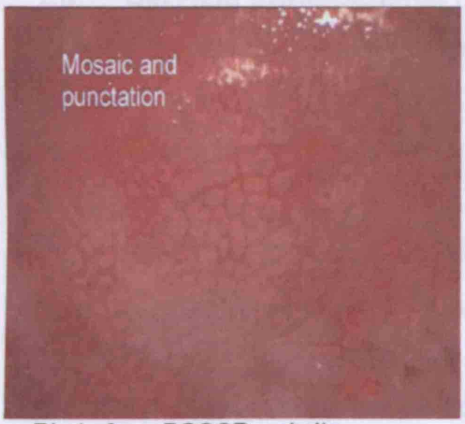



Photo from BSCCP website

This figure shows mosaicism seen at colposcopy of a woman with CIN II after application of acetic acid.

### 1.2.8 Technique

See Appendix 1

### 1.2.9 Referral for colposcopy

Under the 1997 NHSCSP guidelines, referral for colposcopy was recommended after one smear showing moderate or severe dyskaryosis or glandular abnormality, after two smears showing mild dyskaryosis or after three smears showing borderline nuclear change. Referral is immediate when cervical cancer is suspected. Referral can be considered after one mildly dyskaryotic smear if the woman is unlikely to comply with follow up.

Following treatment, colposcopy is recommended if the follow up smear shows dyskaryosis, even mild. If the treatment is excisional and the margins are doubtful or the diagnosis is microinvasion or high grade CGIN colposcopy as well as cytology is recommended.

While colposcopy is a straightforward and generally painless examination, referral for colposcopy can cause significant anxiety to the individual woman (Marteau 1990, McDonald 1989).

These guidelines were updated in 2004 but the above guidelines were in force at the time of the work for this thesis (NHSCSP 2004).

## 1.3 Cervical intraepithelial neoplasia

Prior to the development of invasive cervical cancer, changes occur in the cervical epithelium described as dysplasia (Richart 1973). These changes may be detected cytologically or histologically.

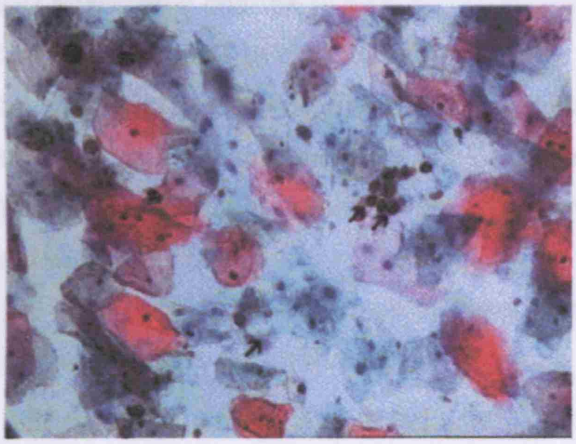
### 1.3.1 Cytology

Cytologists examine exfoliated cells from the cervix and describe morphological changes in the cells' structure. The normal smear consists of a thin layer of cells with small pyknotic, hyperchromatic nuclei. As the cells become abnormal the nuclei change in size and shape. They become larger with broad variation in size and shape, hyperchromasia, irregular chromatin distribution and an irregular nuclear outline. The

nuclear:cytoplasmic ratio is reduced. Mitoses become increasingly common. These changes are progressive with the degree of abnormality of the cell.

*Figure 1.3* Smear test

Photo from BSCCP website



This figure shows a smear demonstrating moderate dyskaryosis; while some normal cells are shown with small hyperchromatic (dark coloured) nuclei, other cells have larger nuclei with irregular outlines.

The original method of describing smears was the Papanicolaou grading system. This was criticised for a lack of equivalent diagnostic histopathologic terminology, a perceived lack of reproducibility between different cytologists and because of a lack of clarity in guiding clinical management. It was felt not to reflect up to date understanding of cervical/vaginal neoplasia (Lundberg 1989). Further classifications were introduced.

**(a) BSCC classification**

The British Society for Clinical Cytology issued a system of classifying cervical smears in the late 1980s, which attempted to correlate cytology with histological findings in the cervical epithelium. The intention was to mimic the histological classification of CIN I-III with a cytological classification of dyskaryosis. The five grades of smear recommended for use were

1. Unsatisfactory for analysis (with reason stated)
2. Negative

3. Nuclear changes bordering on mild dyskaryosis
4. Dyskaryosis: mild, moderate and severe
5. Malignant cells suggestive of invasive cancer; squamous and adenocarcinoma

The BSCC are currently reviewing their terminology to take into account HPV associated change.

**(b) The Bethesda classification**

The National Cancer Institute developed their scheme in the late 1980s and early 1990s to improve communication between the cytologist and the clinician in relation to patient management. It was recommended that each report include

1. a statement of the adequacy of the specimen for diagnostic evaluation
   a) satisfactory for interpretation
   b) less than optimal
   c) unsatisfactory with an explanation e.g. partially obscured by blood
2. a general categorisation of the diagnosis
   a) within normal limits (described cytologically)
   b) other
3. a descriptive diagnosis
   a) infection; fungal, bacterial, protozoan or viral
   b) reactive and reparative changes; inflammation, effects of IUCD
   c) epithelial cell abnormalities
      (i) squamous cell
         - Atypical squamous cells of undetermined significance (ASCUS) with recommendation for follow up or further investigation
         - Cellular changes suggesting
            a low grade squamous intraepithelial lesion (LSIL)
            a high grade squamous intraepithelial lesion (HSIL)
            squamous cell carcinoma
      (ii) glandular cell
         - unexpected presence of endometrial cells
         - atypical glandular cells of uncertain significance

- adenocarcinoma

d) non-epithelial malignant neoplasm
e) hormonal evaluation of vaginal smears

The diagnostic terms are LSIL and HSIL corresponding broadly to borderline and mild dyskaryosis (LSIL) and moderate and severe dyskaryosis (HSIL).

There are clear comparisons between the Bethesda classification and the BSCC system. However differences do exist and in addition it has been shown that interpretation of cytology varies internationally. For example, British pathologists interpreted smears as more severely abnormal than their American or Scandanavian counterparts (Scott 2002).

### 1.3.2 Histology

Histopathologists examine samples of cervical epithelium taken as directed punch biopsies or as excisional samples such as knife cone or LLETZ biopsies. Richart described a concept of cervical precancer; cervical intraepithelial neoplasia (CIN) that dysplastic changes represented a spectrum of severity of the same basic change (Richart 1973). This can be used to classify cervical dysplasia.

CIN I (mild dysplasia)

Upper two thirds of the epithelium exhibits good differentiation
Minor nuclear abnormalities
Few mitotic figures confined to the basal one third.

CIN II (moderate dysplasia)

Upper half of the epithelium is well differentiated
Moderate nuclear abnormalities
Mitotic figures in the basal two thirds

CIN III (severe dysplasia)

Differentiation confined to the superficial one third of the epithelium or there may be a complete absence of any differentiation.
Nuclear abnormalities marked and throughout the full thickness
Mitotic figures numerous at all levels.

*Figure 1.4* CIN II

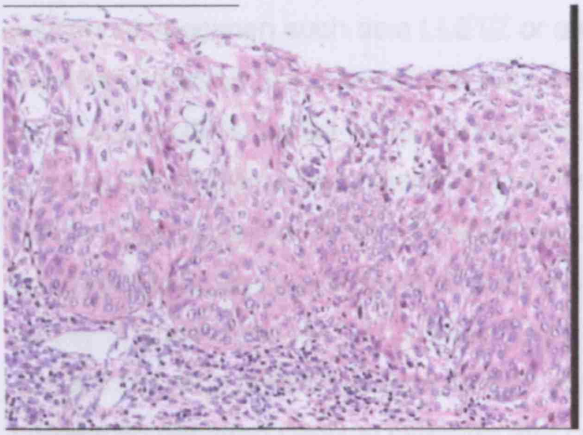


Photo from the BSCCP website

This picture shows a section of tissue showing CIN II. The upper half of the epithelium remains well differentiated. Mitotic figures appear in the basal two thirds.

It is clear that there is some correlation (but not complete concordance) between CIN I-III and mild, moderate and severe dyskaryosis in the BSCC cytology classification system. Under the Bethesda classification CIN is replaced by squamous intra-epithelial lesion (SIL). LSIL corresponds to CIN I and HSIL to CIN II and III. There is a distinct clinical division between LSIL and HSIL (or CIN I and CIN II/III). The management of HSIL is clearer and treatment is usually recommended. The management of LSIL is a matter for debate (discussed in 1.3.8) and it groups together a range of patients whose treatment provides a clinical dilemma as their condition is likely, but not certain, to resolve untreated. The BSCC has a working group discussing terminology which is considering a revision of their classification to high and low grade disease. Because of the clinical division, the Bethesda classification of HSIL and LSIL were used in this study rather than the current BSCC classification.

### 1.3.3 Diagnostic accuracy

The gold standard for diagnosis of a cervical lesion is histology of an excisional specimen such as a LLETZ or a cone biopsy. However inter-observer variation between pathologists is significant and agreement can be as low as 65% (Ismail 1989). Comparisons of alternative diagnostic techniques usually use excisional biopsy histology as a reference.

Heatley et al (Heatley 1998) examined the correlation between grade of dyskaryosis on cervical smears and the grade of CIN on directed punch biopsy or cone biopsy in 107 women. They concluded that there was 63% exact correlation between punch biopsy and cone biopsy but only 49% correlation between cervical cytology and cone biopsy. These levels of correlation were equivalent to those found in Matsuura's study (Matsuura 1996). However a review by Nanda revealed a lack of unbiased data in assessing the accuracy of cervical cytology and suggested an average sensitivity of cytology to detect cervical cancer of only 51% (Nanda 2000). Buxton's study of paired punch biopsies and excisional biopsies showed only 46% agreement between diagnosis (Buxton 1991). Women whose cytology suggests low-grade disease may have HSIL in up to 40% of cases (Jones 1992). Jones showed that regular audit of mild dyskaryosis or borderline smears where the subsequent histology showed HSIL improved the diagnostic accuracy of cytology in a particular laboratory (Jones 1996). This poor specificity of cytology has lead to a practice of repeated smears and early colposcopy for women whose smears suggest low grade disease. It has also prompted the search for alternative, more specific screening markers such as HR HPV, constitutional factors and immunological responses.

Reid identified wide inter-operator variation between colposcopic diagnoses and developed an index to try to improve diagnostic reliability (Reid 1985). The level of diagnostic accuracy is better at colposcopy in invasive cancer and HSIL than in low-grade disease. A meta-analysis of the accuracy of colposcopic diagnosis suggests that in differentiating CIN of all grades from normal cervix, colposcopy has 96% sensitivity and 48% specificity. When differentiating between normal or LSIL and HSIL or invasive cancer, sensitivity is 85% and specificity 69% (Mitchell 1998). A

combination of cervical cytology, colposcopy and histology improves diagnostic accuracy. However this is time consuming and invasive and reflects the need for a more accurate screening test.

### 1.3.4 Incidence of CIN

In 2002-2003 4% of adequate smears showed mild dyskaryosis, 2.1% moderate dyskaryosis and 0.7% severe dyskaryosis (DOH 2003).

### 1.3.5 Aetiology of CIN

The involvement of an infectious agent in the development of cervical disease was first suggested by epidemiological studies identifying sexual activity at a young age and promiscuity as risk factors for cervical cancer (Rotkin 1967). Initial studies investigated the role of herpes simplex virus but the link to human papillomavirus was made by zur Hausen in 1976 who pointed out that genital warts (condylomata accuminata) show an identical epidemiological pattern as cervical cancer and postulated that a papillomavirus could also be involved in the development of cervical cancer (zur Hausen 1976). In the meantime, cytologists frequently reported the presence of virus related cellular abnormalities called koilocytes in the smears of women with mainly mild morphological changes, where condylomas could not be detected. Over 30 different genital HPV's have been isolated with different oncogenic potential, about 15 of which have been isolated from high-grade cervical dysplasia or cervical cancers (de Villiers 1989).

Cigarette smoking is linked to the development of CIN. It has been suggested that sexual behaviour is a confounding factor in this link but case control studies adjusted for sexual variables have generally shown an independent association for smoking (Winkelstein 1990).

### 1.3.6 Natural History

Richart's original concept of CIN as a continuum of disease suggests a progressive nature with abnormalities progressing from mild to severe and on to invasive cancer (Richart 1973). This concept is supported to some extent by observations of CIN and cervical cancer. The incidence of CIN is highest at least a decade before the incidence of invasive cancer. Women with CIN develop invasive cancer more frequently than those with normal epithelium. Early invasive cancer is often surrounded

by a border of CIN. The risk factors for CIN and cervical cancer are common.

However the numbers of cases of dysplasia is far in excess of the number of cases of cervical cancer even without treatment illustrating that the majority of lesions will not progress to cancer. Numerous studies have attempted to quantify the risk of progression from CIN to cancer and from low grade CIN to high grade CIN. A range of results represents variation in method and interpretation. Studies vary in their starting point from a single abnormal smear to multiple repeated abnormal smears and in their length of follow up from one to eighteen years. Endpoints differ from development of cervical cancer to decision to treat based on clinical observation to an arbitrary cut off point in time. There is a lack of uniformity in the diagnostic techniques (cytology, histology and colposcopy) used and the interpretation of the results of these techniques. Currently, in developed countries, the natural history of CIN is difficult to study as treatment usually follows diagnosis of high-grade lesions, interrupting the natural history of the disease. It is ethically difficult to proceed in an alternative way.

*Table 1.1* Studies of natural history of CIN

| Author Year (Number of patients) | Diagnosis | % Regression | % Persistence | % Progression | Follow up (years) |
|---|---|---|---|---|---|
| Koss 1963 (93) | CIN I-II<br>CIS | 39<br>25 | 15<br>61 | 42<br>6 | 0.5-7<br>3 |
| Fox 1967 (278) | Mild-moderate dysplasia | 31 | 9 | 60 | 10 |
| Hall 1968 (206) | Slight-<br>Moderate<br>Severe dysplasia | 62<br>33<br>19 | 24<br>49<br>48 | 13<br>18<br>33 | 1-14 |
| Barron 1970 (557) | All dysplastic | 6 | 28 | 66 | 6.3 |
| McIndoe 1984 (1028) | CIS | | 13.8 | 23.6 | 4-25 |
| Campion 1986 (100) | CIN I | 11 | 67 | 26 | 2.6 |
| Mitchell 1986 (846) | CIN I | 82 | | 18 | 6 |
| Nasiel 1986 (555) | Slight dysplasia | 62 | 22 | 16 | 4.1 |
| Syrjanen 1987 (513) | Koilocytosis | 24.8 | 59.8 | 14.1 | 2.1 |
| Kataja 1989 (532) | Koilocytosis<br>CIN I<br>CIN II<br>CIN III | 42<br>31<br>34<br>21 | 50<br>57<br>42<br>29 | 8<br>12<br>20<br>50 | 3.3 |
| Courtial 1991 (146) | LSIL | 65 | 35 | | 1-2 |
| Carmichael 1991 (525) | Mild dysplasia | 77 | 22 | 7.8 | 0.5-5 |
| Montz 1992 (37) | LSIL | 78.3 | 18.2 | 3.4 | 1 |
| Syrjanen 1992 (528) | CIN I<br>CIN II<br>CIN III | 58<br>53<br>14 | 22<br>24<br>15 | 15<br>20<br>69 | 10 |
| Remmink 1995 (342) | CIN | 83 | | 17 | 3 |
| Katase 1995 (87) | CIN I-II | 50.6 | 44.8 | 4.6 | 5 |
| Furber 1997 (738) | LSIL | 80 | 20 | | 2 |
| Matsuura 1998 (43) | LSIL | 48.8 | 51.2 | | 5 |
| Duggan 1998 (342) | CIN I | 62.7 | 18.6 | 18.6 | 2-3 |

| | | | | | |
|---|---|---|---|---|---|
| Paraskevaidis 2002 (360) | HPV+/-CIN I | 56 | 33 | 11 | 10 |

(Barron 1970, Campion 1986, Carmichael 1991, Courtial 1991, Duggan 1998, Fox 1967, Furber 1997, Hall 1968, Kataja 1989, Katase 1995, Koss 1963, Matsuura 1998, Mitchell 1986, Montz 1992, Nasiell 1986, Paraskevaidis 2002, Remmink 1995, Syrjanen 1987, Syrjanen 1992)

A review of previous studies by Oster in 1993 (Ostor 1993) suggested the likelihood of regression to normality from CIN I was 60%, CIN II 40% and CIN III 33%. The likelihood of persistence for the three grades was 30%, 40% and 55% and the likelihood of progression to CIN III was 10% from CIN I and 20% from CIN II. The risk of invasion was 1% from CIN I, 5% from CIN II and 12% from CIN III.

A historical cohort of Canadian women whose smear histories were recorded at a major cytopathological laboratory provided the opportunity to study the natural history of the disease in an era (1962-1980) during which cervical squamous lesions were managed conservatively (Holowaty 1999). The results showed that the risk of progression from mild dysplasia to severe dysplasia or worse was only one per cent per year and from moderate dysplasia 16% within two years and 25% within five years. The rate of regression to a normal smear within two years was 44% for mild dysplasia and 33% for moderate dysplasia. During the same period, McIndoe et al observed 948 patients with carcinoma in situ for periods of 5-28 years. Of these 673 women had cone biopsy or amputation of the cervix prior to the observation period. Those whose cytology reverted to normal had a 1.5% risk of developing invasive cervical cancer compared to a 22% chance for those with ongoing abnormal cytology (McIndoe 1984).

### 1.3.7 Effect of punch biopsy on natural history

The diagnostic process itself may have an effect on the natural history of cervical disease. It is possible that a colposcopically directed punch biopsy may remove all the abnormal epithelium. The sites will re-epithelialise with normal epithelium, giving the impression of regression at follow up. Biopsy also induces an inflammatory response and the

subsequent healing response may also affect the natural history of the disease. In LLETZ specimens taken after recent punch biopsies pathologists often see an inflammatory response. Interpretation of studies examining this issue is difficult. Those prospective studies where biopsy was not undertaken risk diagnostic inaccuracy since they use two diagnostic parameters instead of three while those where biopsies are taken cannot be regarded as truly observational. The studies also vary according to follow up interval; if short the full effect of biopsy may not have occurred, if long any change may be more related to the passage of time than the biopsy. Koss's original work suggested regression was more common after tissue biopsy (Koss 1963). Subsequently Chenoy *et al* and Youkeles *et al* have shown that this is not necessarily the case (Chenoy 1996, Youkeles 1976).

### 1.3.8 The management of women with low grade smears

The management of women whose smears show moderate dyskaryosis or above is almost universally agreed to be immediate referral for colposcopy and treatment. The management of women whose smears show mild dyskaryosis or borderline change remains controversial. The balance must be struck between prompt diagnosis and treatment for those women who require it without the considerable cost and workload for colposcopy clinics and anxiety for women whose chance of significant disease is low. Studies investigating management of low-grade smears have come to a variety of conclusions. There is variation in recruitment criteria between mild and moderate dyskaryosis, mild dyskaryosis and borderline nuclear change, mild dyskaryosis alone and single or repeated abnormalities at recruitment. The majority of studies are retrospective using variation in local referral practice to compare outcomes. There is variation in end points between an arbitrary cut off point in time, the development of HSIL or discharge from the clinic.

*Table 1.2* Studies of the management of low-grade smears

| Author Year (number of patients) | Type of study Recruitment cytology | Results | Recommendations |
|---|---|---|---|
| Walker 1986 (228) | Retrospective Mild dyskaryosis | Poor correlation between low grade referral smear and histology | Reduced reliance on cytological surveillance |
| Soutter 1986 | Review Mild atypia | CIN present in 37% of mild atypia and 49% of mild dyskaryosis | Repeat cytology at 3/12 in mild atypia, refer if abnormal |
| Giles 1989 (200) | Retrospective Mild dyskaryosis | At colposcopy: 27% normal, 29% CIN I, 33% CIN II/III Repeat cytology 24% false negative | Repeat cytology is safe practice |
| Robertson 1988 (1781) | Retrospective Mild dyskaryosis | 10 cases of invasive cancer 3 in defaulters. Repeat smears improve diagnosis | Cytological surveillance. Biopsy for persistent abnormality |
| Kirby 1992 (500) | Retrospective Mild/moderate dyskaryosis | At end of follow up 60% normal, 37% biopsied, 19% CIN III | Cytological surveillance preferred over immediate colposcopy |
| Jones 1992 (278) | Retrospective Mild dyskaryosis | Cytological surveillance; 29% normal, 23% CIN III. Immediate colposcopy; 17% normal, 20% CIN III | Cytological surveillance recommended |
| Cooper 1992 (337) | Retrospective Mild dyskaryosis | 34% regression, 53% biopsied, 38% ongoing follow up | Cytological surveillance. Biopsy for persistent abnormality |
| Hunt 1994 (221) | Retrospective Mild atypia, Mild dyskaryosis | Mild atypia; 8% treatment, Mild dyskaryosis; 51% treatment, 27% CIN II/III | Lack of consistent management. Randomised controlled trial |
| Flannelly 1994 (902) | Prospective Mild/moderate dyskaryosis | At end point 30% normal 25% CIN III. Increased default rate with length of follow up | Immediate colposcopy |
| Shafi 1997 (353) | Prospective randomised Borderline, mild dyskaryosis | Cytological follow up; 20% normal, 25% CIN III Immediate treatment; 0.6% normal, 24% CIN III 21% default | Immediate colposcopy |
| Teale 2000 (566) | Prospective Borderline, mild dyskaryosis | 54% regression, 25% persistence, 21% progression | Cytological surveillance safe after normal colposcopy and negative smear |

(Cooper 1992, Flannelly 1994, Giles 1989, Hunt 1994, Jones, Jenkins 1992, Kirby 1992, Robertson 1988, Shafi 1997, Soutter 1986, Teale 2000, Walker 1986)

All these studies used biopsies to confirm diagnoses at the endpoints rather than relying on cytology and colposcopy alone. Walker and Soutter showed a poor correlation between cytology reported as low grade and subsequent histology (Soutter, Wisdom 1986, Walker, Dodgson 1986). Giles reported a false positive rate of 24% for repeat cytology but found that low grade lesions were more commonly missed (Giles, Deery 1989). In the largest study by Robertson et al, this poor correlation between low grade smears and histology was also noted but was improved by repeated cytology. In this study there were 10 cases of invasive cervical cancer, three of which occurred after default from follow up (Robertson, Woodend 1988). Jones compared policies of cytological surveillance and immediate colposcopy by retrospectively comparing areas with different policies. In patients referred immediately for colposcopy, there were no cases of CIN III at 24 months. In those patients undergoing cytological surveillance, 33% were referred for colposcopy during 24 month follow up and of the remaining 66%, 18% had CIN II/III. Only 67% of these were identified in cytology (Jones, Jenkins 1992). Cooper identified one case of microinvasive cervical cancer within 12 months of a single mild dyskaryosis smear and a 46% rate of CIN II/III in women referred to colposcopy with persistant or progressive abnormal cytology (Cooper, Kirby 1992). These retrospective studies confirm that cytological surveillance reduces the referral rate of women with a normal cervix to colposcopy. However the correlation between low grade cytology and histological diagnosis is low and cytological surveillance does not appear safe in isolation when cytological abnormalities persist. Flannelly's prospective study showed cytological surveillance was associated with 75% default over 24 months (Flannelly, Anderson 1994). Shafi showed that women randomised to cytological surveillance and immediate colposcopy had similar rates of CIN II/III at 24 months while the

cytological surveillance group had reduced incidence of CIN I (25 to 13%) and increased normal cervix at colposcopy (0.6 to 20%). However they identified one case of microinvasive cervical cancer at six months from a mild dyskaryosis smear and concluded that cytological surveillance alone was sub-optimal (Shafi, Luesley 1997). Teale developed a suggested management algorithm for management of women with low grade smears after colposcopy (Teale, Moffitt 2000).

Johnson suggested a decision analysis tool to help compare the expected mortality and cost of each policy (Johnson 1993). The spread of results and recommendations show that a clear consensus on the management of mildly abnormal smears has not been reached. Most recent studies into the optimum management of low-grade smears have focused on HPV as a triage.

The 2004 NHSCSP guidelines recommend referral for colposcopy after three smears showing borderline change. Persistent borderline change is a risk factor for developing cervical cancer (Hirschowitz 1992). Immediate referral is recommended for women with mild dyskaryosis but repeat cytology is stated to be acceptable. This recommendation is based on the findings that 40% of women whose smears show mild dyskaryosis may have HSIL (Soutter, Wisdom 1986) and as few as 30% of women will experience disease regression to a normal smear (Flannelly, Anderson 1994). In addition immediate referral is more cost effective than cytological follow up (Johnson, Sutton 1993). Following two smears showing mild dyskaryosis referral is mandatory (NHSCSP 2004).

### 1.4 Human Papillomavirus

Human papillomavirus (HPV) is a double stranded, non enveloped circular virus of about 8000 base pairs surrounded by a capsid and measuring 40-55nm in diameter. Study of HPV has been hampered by the inability to propagate these viruses in cell culture as they depend on the differentiation of squamous epithelium *in vivo*. There are early (E) and late (L) open reading frames (ORFs) – DNA segments that encode proteins which show similar organisation. E1 and 2 code for viral DNA replication and control of viral transcription. E6 and 7 are essential in the

process of HPV induced cellular immortalisation and transformation. L1 and 2 code for capsid proteins. Definition of new HPV types is based on less than 90% homology in the ORFs E6, E7 and L1 than any other known HPV type.

HPV types can be divided into high risk and low risk groups according to whether they are associated with malignant or benign disease.

*Table 1.3* HPV types

| HPV types | Lesions |
|---|---|
| 6 | condyloma acuminata, CIN |
| 11 | laryngeal papilloma, CIN |
| 13 | focal epithelial hyperplasia |
| 16 | CIN, cervical carcinoma |
| 18 | " " " |
| 30 | CIN, laryngeal carcinoma |
| 31 | CIN, cervical carcinoma |
| 32 | Focal epithelial hyperplasia, oral papilloma |
| 33 | CIN, cervical carcinoma |
| 34 | CIN |
| 35 | CIN, cervical carcinoma |
| 39 | CIN, cervical carcinoma, PIN |
| 40 | CIN, PIN |
| 42 | CIN, vulvar papilloma |
| 43 | CIN, vulvar hyperplasia |
| 44 | CIN, vulvar condyloma |
| 45 | CIN, cervical carcinoma |
| 51 | " " " |
| 52 | " " " |
| 53 | normal cervical mucosa |
| 54 | condyloma acuminata |
| 55 | Bowennoid papulosis |
| 56 | CIN, cervical carcinoma |
| 57 | CIN, inverted papilloma |
| 58 | CIN |
| 59 | VIN |

CIN, PIN, VIN: cervical, penile and vulvar intraepithelial neoplasia respectively. Modified from de Villiers 1989.

### 1.4.1 Detection of HPV

It is possible to detect viral infections by virus culture, serology (antibody response) and identification of virus particles, antigen or nucleic acid. HPV cannot be cultured in vitro and the diagnostic tools in current use are all based on nucleic acid detection by hybridisation. This involves the linking of two complementary single strands of DNA to form a double

stranded hybrid. A labelled probe of purified HPV DNA or HPV type specific oligonucleotide will link to HPV DNA in the sample. The label allows detection of the PCR products by visualisation using number of different techniques including autoradiography (if radioactive), fluorescence dyes, enzyme immunoassay or immunohistochemistry staining.

Hybridisation depends on the extent of similarity between the probe and the target DNA and on the reaction conditions. By varying these factors the stringency of the hybridisation may be determined i.e. at high stringency only exactly homologous DNA will form hybrids whereas at low stringency a range of similar DNA will also hybridise.

In general methods either detect HPV DNA directly or following amplification by polymerase chain reaction.

**1.4.1a DNA Direct detection**

Southern Blot analysis

Target HPV DNA is purified and digested with specific restriction enzymes. The fragments are separated on the basis of size usually by electrophoresis and blotted onto a DNA binding filter. They are then hybridised with HPV type specific probes. Each HPV type has a specific restriction enzyme digestion pattern and results can be compared to standard patterns.

The technique requires relatively large amounts of DNA (10μg if there are low copy numbers of HPV per cell) and can therefore only be used with at least small tissue samples such as punch biopsies. It is also labour intensive. It can identify whether the HPV DNA is episomal or integrated; integrated DNA is more often found in cervical cancer while episomal DNA is found in benign lesions. It is a sensitive (1pg HPV DNA per 10μg genomic DNA) and specific technique and has been regarded as the gold standard of HPV detection.

Dot blot analysis/ViraPap

In these methods DNA is fixed directly onto a membrane without prior separation. Smaller amounts of DNA are required (0.3-1μg). Dot blot analysis makes use of DNA probes while ViraPap uses RNA probes. The

technique requires high stringency and sensitivity of 0.5-1pg of HPV DNA per 1μg genomic DNA can be reached. Initially these techniques utilised radioactive probes.

Hybrid capture

This technique has largely surpassed the dot blot technique and has been approved by the Food and Drug Administration in the USA. HPV DNA is hybridised in solution with HPV RNA probes. The DNA/RNA hybrids are captured in a tube coated with antibodies against the hybrids. These antibodies are specific for the hybrids and will not capture double stranded DNA or single stranded molecules. The bound hybrids are visualised using colormetric or chemoluminescence based detection methods. This test is available as a standardised kit format and has comparable sensitivity with dot blot analysis. The second-generation kit has sensitivity approaching that of PCR. The drawback is the lack of information about specific HPV types as the test hybridises with a mixture of probes for the oncogenic HPV types.

Filter *In situ* Hybridisation (FISH)

Unprepared cervical scrapes are spotted onto a membrane and the DNA is denatured. HPV specific probes are used for hybridisation. FISH has half the sensitivity of Southern blotting and is subject to high background readings.

*In situ* Hybridisation (ISH)

In this technique the orientation of biopsy specimens is preserved in order to allow localisation of the infected cells. The specimen is treated with partial digestion of the cellular protein and labelled probes are added. This technique is not useful for screening because biopsies are required. However, because of the ability to locate where the viral conscripts are transcribed in comparison to the cervical disease, it has contributed to the understanding of the role of HPV in the pathogenesis of cervical cancer.

**1.4.1b DNA detection following amplification with PCR**

Polymerase Chain Reaction (PCR)

This technique allows *in vitro* amplification of a piece of target HPV DNA. The target DNA is denatured at 94°C. Oligonucleotide primers (small

single strands of complementary DNA) are annealed at 37-70 °C. DNA is synthesised by primer extension of the free 3' OH end using a thermostable DNA polymerase at 72 °C. Thirty to 40 cycles of amplification achieves an exponential increase in the amount of HPV DNA, which can be detected after gel electrophoresis. This technique is exquisitely sensitive and therefore prone to contamination with environmental DNA, PCR products and cloned plasmids unless rigid laboratory discipline is adhered to.

HPV Type specific PCR

Primers are specific to individual HPV types. They can be used to identify a particular HPV type under study or in combination to type multiple HPV types. Each primer is selected so different amplified fragment sizes are generated for different HPV types and thus identified independently.

General or consensus Primer mediated HPV PCR

The use of primers, which amplify a broad spectrum of HPVs in one reaction, reduces the number of PCRs required. Different approaches are available to overcome the lack of perfectly matched areas in multiple HPV types.

Lower stringency of primer annealing accepts mismatch between primers and target DNA. Degenerate primers with nucleotide differences at several positions allow annealing with multiple target DNAs. Primers may contain inosine at certain ambiguous base positions. The MY09/11 consensus primer uses degenerate bases, the GP5+/6+ primer system uses a forward and reverse primer that is complementary with an area of high homology between various HPV types and low stringency annealing.

### 1.4.2 Links between HPV and cervical disease

The first connection between cervical cancer and an infectious agent was made over 100 years ago. While herpes simplex virus was initially studied, no HSV DNA was found in cervical tumours and it became apparent that HPV was the likely agent (zur Hausen 1994). It is now clear that high-risk HPV types are present in virtually all squamous cell cervical cancers (Walboomers 1999). Bosch carried out a worldwide study of the prevalence of HPV in cervical cancer (Bosch 1995). The rate of HPV

infection in women with invasive cervical cancer was 92.9% with consistency internationally from 75-100%. Further testing by histological confirmation of the cancer diagnosis and repeated PCR on the apparently negative specimens found no evidence of HPVI in less than 5%. However HPV infection is present in as many as 16-20% of normal smears (Hatch 1995). Thus not all HPVI leads to cervical disease.

HPV infects the genital epithelium via sexual contact. The viral genome forms an episome in the basal cell layer which may remain latent or establish an infection of the parabasal cells with subsequent morphological expression in differentiating squamous cells (Jenson 1991). As the viral capsid assembles, degenerative changes in the cytoplasm and nuclei occur known as koilocytic atypia (Jenson 1987). In LSIL the virus remains in the episomal form with the E6 and E7 ORFs physically intact. In HSIL and carcinoma the HPV DNA is partly integrated into the host DNA. A disruption of the E1-E2 region is required for HPV genome integration. This event results in an increased expression and stabilisation of the E6 and E7 transcripts. The E6 protein from high-risk HPVs binds cellular p53, promoting the degradation of p53 by the cellular ubiquitin proteolysis system. The E7 protein interacts with pRB (retinoblastoma) and inactivates this cellular protein. As a consequence, E2F transcription factor is released from pRB-E2F complex, leading to transcriptional activation of several genes involved in cell proliferation. Such interactions of HPV E6 and E7 proteins interfere with two major pathways involved in the control of the cell cycle.

Most infection with high risk HPVs is transient but persistent infection appears to be required for cervical disease (Remmink, Walboomers 1995). It seems the prevalence of infection is around 10% in 20-24 year olds and 3% in over 30 years olds (Meijer 1998).

Studies investigating the prevalence and natural history of HPV have varied in their conclusions, partly due to different HPV detection methods and media being tested (smears, biopsies, fresh or archived material). Table 1.4 summarises these studies.

*Table 1.4* Studies examining the incidence and effect of HPV infection

| Author Year (number of patients) | Recruitment criteria | Method of HPV detection HPV types studied | Results |
|---|---|---|---|
| De Villiers 1987 (9295) | Normal cytology CIN | FISH 6, 11, 16, 18 | Normal cytology; 10% HPV positive <50 yrs, 5% >50 CIN; 35-40% HPV positive |
| De Villiers 1992 (11667) | Normal cytology | FISH 6,11,16,18 | 8.8% HPV 16/18 positive, 11% <55 yrs, 3.2% >55yo. In women developing cancer 63.2% HPV positive at recruitment |
| Koutsky 1992 (241) | Normal cytology | PCR 16,18 | Incidence of HSIL 28% if HPV positive and 3% if HPV negative at 2 years |
| Cuzick 1994 (133) | Abnormal cytology | PCR 16, 18, 31, 33, 35 | CIN III; 59% high levels of HPV 16, 84% high levels of 16, 18, 31, 33 or 35 |
| Remmink 1995 (342) | Abnormal cytology | PCR 6,11,16,18,31,33 and others | Progression to CIN III only occurred with continuous type specific HPVI |
| Flannelly 1995 (62) | Mild/ moderate dyskaryosis | PCR 16 | High levels of HPV 16 associated with CIN II/III at LLETZ |
| Duggan 1997 (537) | Cytology suggesting CIN I | PCR 6,11,16,18,31,33, 35 | No difference in rate of HPVI in patients with CIN I or CIN II/III 16/18 28%, 31/33/35 10%, 6/11 2% |
| Ho 1998 (608) | College student volunteers | PCR, Southern blot HPV DNA | Cumulative incidence over 36/12 of HPVI 43%. Mean duration of infection 8/12 |

| | | | |
|---|---|---|---|
| Cruickshank 1999 (304) | Mild dyskaryosis | PCR 16, 18 | Using HPV testing in combination with repeat cytology results in 88% referral to colposcopy |
| Wallin 1999 (118) | Normal cytology, later developing cervical cancer | PCR HPV DNA | 30% HPVI in women with normal cytology who later developed cervical cancer, 3% HPVI in age matched controls. Persistence of type specific HPV |
| Nobbenhuis 1999 (353) | All dyskaryosis | PCR High risk HPV types | Persistent HPVI required for progression to CIN III |
| Manos 1999 (995) | ASCUS | PCR HPV DNA | 6.7% progression to HSIL/ cancer. Positive HPV test 89.2% sensitivity, 64.1% specificity. Equivalent to repeat cytology |
| Ylitalo 2000 (478) | Normal cytology later developing cervical cancer | PCR 16 | High viral load levels detected in cytologically normal smears of women who went on to develop cervical cancer |
| Ylitalo 2000 (484) | Normal cytology later developing cervical cancer | PCR 16 | HPVI on two consecutive smears increases risk of cervical cancer 30 fold. Mean incubation period from infection to cancer 7-12 years |
| Solomon 2001 (3488) | ASCUS | HC II High risk HPV | 5.1% CIN III. HPV test triage 96.3% sensitive with 56% referred to |

| | | | |
|---|---|---|---|
| | | | colposcopy. Repeat cytology 85.3% sensitive with 58.6% referred |
| Denise Zielinski 2001 278 | Borderline/ mild dyskaryosis | HC II | 96.3% sensitivity, 60.2% specificity for CIN II/III at first visit. |
| Rebello 2001 (333) | Persistent borderline/ mild dyskaryosis | HC II | 93% sensitivity, 55% specificity 94% negative predictive value for CIN II/III |
| Kjaer 2002 (10758) | Normal cytology, LSIL, HSIL | PCR High risk and low risk HPV | Clearance of HPVI 71% with normal cytology, 40% with LSIL, 24% with HSIL |

(Cruickshank 1999, Cuzick 1994, de Villiers 1987, de Villiers 1992, Denise Zielinski 2001, Duggan 1997, Flannelly 1995, Ho 1998, Kjaer 2002, Koutsky 1992, Manos 1999, Nobbenhuis 1999, Rebello 2001, Remmink, Walboomers 1995, Solomon 2001, Wallin 1999, Ylitalo 2000, Ylitalo 2000)

The evidence for use of HPV testing as a primary screening tool was reviewed by Cuzick et al (Cuzick 1999). They concluded that the evidence was not strong enough at that time to justify the introduction of HPV testing into the NHS screening programme. Their primary reasons were the low specificity in young women, the lack of a large scale trial with reduction in cancer incidence as an endpoint and the uncertainty over the safety of a negative result. They identified potential uses in the over 30s, in borderline/ mild dyskaryosis and in post treatment surveillance. The studies above show that in the rate of HPV infection is high in women under 30 resulting in a low specificity but over 30 the background rate is much lower. The studies above examining use of HPV testing as a triage for low grade smears have shown it to be at least as

sensitive as repeat cytology. The initial study into liquid based cytology included an arm for assessing HPV testing in this role. The HART study investigated the use of HPV testing as a primary screening tool. They performed HPV testing at the time of cervical smears and investigated women who were high risk HPV positive either by immediate colposcopy or by repeat HPV test and cytology at 6 months. They showed cytology of mild dyskaryosis or above to have 70.1% sensitivity for HSIL with 98.6% specificity while HPV testing had sensitivity of 96% with specificity of 94.4%. They included only women over 30 years old where HPV testing has improved specificity. They introduce the suggestion of primary screening with the most sensitive tool, HPV testing followed by the more specific tool, cytology in positive cases. The work could not be applied to women under 30 and a separate algorithm would be required (Cuzick 2003).

## 1.5 Human immune response

The immune system, the means by which humans fight infection is divided into two parts; innate and adaptive responses.

Innate immunity is involved in immediate host defence but has the drawback of being non-specific and may therefore damage normal host tissue. Adaptive response takes longer to develop but is specific to the infecting agent. It also has memory and subsequent exposure to the same agent results in a more rapid response.

### 1.5.1 Innate immunity

At the start of an infection or injury, neutrophils are recruited and activated to the site of injury by the actions proinflammatory mediators, adhesion molecules, chemoattractants and chemokines. Activated neutrophils phagocytose infecting particles and destroy them (Witko-Sarsat 2000).

Complement consists of at least 20 serum glycoproteins which activate in a cascade sequence and include some proteins responsible for regulation and amplification. Complement acts by attacking cell surface membranes resulting in cell lysis. Host cells contain a receptor to inhibit the complement cascade and therefore prevent host cell damage but these receptors are lacking in microbes. Complement also has components

which act by increasing vascular permeability allowing antibodies to permeate tissue and as part of the adaptive immune response by providing targets for antigen presenting cells.

Eosinophils are largely involved in response to parasitic infection. Mast cells (mainly in mucosa and tissue) and basophils (mainly in blood) release highly active enzymes to produce increased vascular permeability, bronchoconstriction and inflammation.

Natural killer cells carry receptors for MHC class I and if they encounter a cell without this molecule, they induce apoptosis. As host cells carry MHC I this protects them from NK cells. In addition they carry immunoglobulin receptors which bind antibody coated cells as part of adaptive immunity. The actions of the innate immune system require exposure to the surface of the infecting molecule and it is therefore more important as a response to extracellular organisms such as bacteria than intracellular organisms such as viruses (Medzhitov 2000).

### 1.5.2 Adaptive immunity

Adaptive immunity is characterised by activated T and B cells. These cells are both produced in an immature form in the bone marrow where B cells remain while T cells migrate to the thymus. The cells develop a huge repertoire of antigen specific receptors intended to combat a broad range of potential pathogens.

B cells are activated by exposure to antigen. They can respond by direct antigen activation to secrete IgM but these responses do not lead to memory and are therefore short-lived. Antigen recognised by B cells can also be internalised, processed and re-expressed on the MHC II molecule. The antigen is then represented to a T2 helper cell which secretes cytokines leading to B cell proliferation and maturation into antibody producing plasma cells. These produce large quantities of antigen specific immunoglobulin, initially IgM but subsequently IgG. Further B cells revert to a resting state and expand the memory population for the same specific antigen so that further infection can be combated with a more rapid production of IgG.

T cells can only recognise antigen presented by self MHC molecules. This presentation occurs as antigen may be produced within the cell such

as in viral or tumour proteins, it may be complexed with MHC I through intracellular processing pathways or it may be taken up by specialised antigen presenting cells including dendritic cells, B cells and macrophages and thus expressed in MHC II molecules. Antigen complexed with MHC I is recognised by CD8 (cytotoxic) T cells and that complexed with MHC II is recognised by CD4 T cells. Thus the type of antigen presentation determines the immune response generated. CD8 T cells act by highly targeted cytotoxic attack while CD4 T cells produce cytokines with a broad range of actions (Parkin 2001).

The actions of the innate and adaptive immune system are coordinated by systems of cellular communication in order to recruit an immune response to a site of inflammation or infection.

**1.5.3 Tumour antigens**

Tumours present antigens to the immune system and can be recognised by the immune system as non-self. These antigens come from a variety of sources.

1. New genetic information introduced by a viral infection
2. Action of carcinogens on oncogenes, which either generate a new protein sequence directly or result in the induction of genes that are normally not expressed
3. Alteration of the cell membrane structure because neoplastic cells lose the ability to synthesize some membrane components
4. Release of antigens normally sequestered in the cell or its organelles when neoplastic cells die.

Intracellular tumour antigens can be complexed by MHC I molecules and presented on the cell surface facilitating their recognition by the host immune system (Robins RA 1987).

**1.5.4 Immune response to tumours**

Tumours provoke an immune response in the host. This is demonstrated by;

1. the invasion of the tumour mass by mononuclear cells
2. tumour specific cytotoxic T lymphocytes and natural killer cells found in patients with cancer

3. altered expression of major histocompatibility complex (MHC) class I and II molecules on the surface of tumour cells (Janaway 1997).

Macrophages can kill specific tumour cells when activated by tumour antigens or by lymphokines. Natural killer cells recognise the low levels of MHC class I on tumour cells and identifies them as non-self. Cytotoxic T cells carry out immunological surveillance and recognise tumour antigen on newly transformed tumour cells (Scott 1991). This immune response is modified by the action of suppressor T cells (Bloom 1992). The balance of cytotoxic T cells and suppressor T cells is influenced by lymphokines. Anti-tumour antibodies may develop particularly against tumours with surface antigens of high density.

However the immune system can fail to control tumour growth for a number of reasons. Some tumours including cervical cancer are poorly immunogenic and MHC expression is down- regulated (Connor 1990). Tolerance may develop to the tumour antigen. The immune response may be inhibited by chemical, physical or viral agents for example in HIV infection where the virus destroys CD4 lymphocytes. Tumours can shed antigen, which binds to tumour specific lymphocytes, preventing tumour recognition by such lymphocytes.

Immune response may also be impaired by an alteration in cytokine production profile. Neoplastic disease, as well as infectious and inflammatory disease, has been associated with dysregulation of the equilibrium between the production of certain type 1 and type 2 cytokines (Clerici 1998).

**1.5.5 Immune response to HPV**

The immune response is vital to protection against HPV. High grade CIN and cervical cancer are more common in women who are therapeutically immunosuppressed (Halpert 1986) and those with symptomatic HIV (Heard 2000). However HPV appears to evade the immune response by a number of mechanisms.

The virus is restricted to the epithelium and therefore does not present to immune system in blood or other tissue (Tindle 2002). The virus does not lyse epithelial cells and is therefore not detected at high levels by

Langerhans cells (antigen presenting cells in the skin) or presented to T cells (O'Brien 2002).

The virus produces early gene products (E1,E2, E5, E6, E7) in the basal keratinocytes. These viral proteins are immunogenic and have been demonstrated to cause rejection reactions when used as vaccines in mice (Tindle 1996). However they are localized in the nucleus and at too low levels or are not effectively presented to the immune system to generate an effective response in natural infection.

Viral infection induces production of interferons. IFN-α and IFN-β activate cellular genes which destroy mRNA and inhibit the expression of viral and some host proteins leading to inhibition of viral replication. They also induce MHC I expression in non infected cells making virus infected cells more susceptible to NK cells and they activate NK cells. IFN-γ induces virus specific cytotoxic T cells.

In addition to its' other effects, HPV 16 E7 protein blocks IFN-α activity preventing its' antiviral immune activity (Barnard 2000). HPV 16 E6 and E7 also inhibit IL-18. This reduces production of IFN-γ and thus cytotoxic T cells (Lee 2001). It is possible that E7 also inhibits NK cell activity as cells expressing E7 are resistant to NK cell lysis in vitro (Routes 2000). E7 also acts by suppressing the function of Langerhans cells as antigen presenting cells (Frazer 1999). These immature Langerhans cells produce tolerance to E7 from CD4+ T cells rather than immune response (Doan 2000).

Late gene products (L1, L2) are expressed in the more distal layers and capsids are formed and shed in epithelial cells at the surface. The levels of L1 and L2 are kept low as they contain codons rarely used in human cells which limits the availability of appropriate tRNA and prevents excess production of these capsid proteins (Schwartz 2000). This reduces the opportunity for host immunity to encounter these proteins.

### 1.6 Cytokines

Cytokines are small molecular weight messengers, proteins or glycoproteins, secreted by one cell to alter the behaviour of itself or another cell. They act on target cells by binding to specific cytokine receptor ligands, inducing second messenger pathways within the cell.

Most are soluble but some are membrane bound, making distinction between cytokine and cytokine receptor difficult (Onishi 1998). Cytokines are produced by virtually all cells and have a wide variety of functions. Their production can lead to cell activation, division, growth and differentiation, migration or apoptosis. They act in a highly complex co-ordinated network and they induce or repress their own synthesis as well as that of other cytokines and cytokine receptors. Many cytokines appear pleiotropic; there is overlap and redundancy between the actions of individual cytokines (Parkin and Cohen 2001).

In an attempt to classify cytokines by cell of origin and function they are divided into broad groupings. Interleukins are produced by leucocytes and have effects mainly on other white cells. Chemokines have chemoattractant activity. Colony stimulating factors have proliferation and differentiation effects on stem cells. Interferons interfere with viral replication (Casciari 1996, Minasian 1992).

Cytokines also distinguish between different groups of T cells. T helper (Th) CD4+ cells orchestrate the immune system; they recognise foreign antigen and activate parts of the cell-mediated immune response to eradicate the pathogen. While they are morphologically indistinguishable, when activated the Th lymphocytes become either Th1 or Th2 cells depending on the cytokines they produce. Th1 cells produce cytokines that induce a cell mediated inflammatory response; interleukin-1 (IL-1), IL-2, interferon gamma (IFN-$\gamma$), and tumour necrosis factor alpha (TNF-$\alpha$). This leads to a pro-inflammatory profile, essential for the prevention of the replication of intracellular pathogens but potentially contributing to the pathogenesis of autoimmune disease. IL-1 and TNF-$\alpha$ are known as primary cytokines as they induce the synthesis of other cytokines and chemokines. Th2 cells produce cytokines that contribute to a B cell mediated humoral response; interleukin-4 (IL-4), IL-5, IL-6 and IL-10. This leads to an anti-inflammatory profile (HayGlass 1996, Kapsenberg 1991). These subsets are well defined in mice while in humans the distinction is less clear cut. There is a Th0 subset, which produce cytokines typical of Th1 and Th2. However it does appear that the overall balance of pro and

anti-inflammatory cytokines influences the outcome of many infectious, autoimmune and malignant diseases. Individuals' immune responses to disease vary and this may reflect variation in levels of cytokine production and therefore the balance of Th1 and Th2 cytokines (Lucey 1996, Witkin 2000).

### 1.6.1 Cytokine gene polymorphisms

Non conservative mutation in the coding region of genes can result in the loss or change in function of the expressed protein as a result of changes in protein structure. These mutations are unusual in cytokine and cytokine receptor genes. Conservative mutations do not affect the amino acid sequence but they may influence protein expression by altering mRNA splicing, mRNA stability and levels of gene transcription. Polymorphisms within the 5'- and 3'- regulatory sequences or introns of genes may have a significant effect on transcription. They may alter the structure of transcription factor binding sites within gene promoters or the structure of enhancers and silencers within introns or at remote regulatory sites. They may alter binding sites within the nuclear matrix for architectural transcription factors, which modulate promoter geometry. Polymorphisms may be substitutions, where a single base is replaced by an alternative, deletions, where a nucleotide is lost from the sequence or insertions, where one or more nucleotides are inserted into the sequence. Many of the reported gene polymorphisms within cytokine genes occur within known or assumed regulatory regions (Bidwell 1998, Bidwell 1999). Thus the variation in levels of cytokine production may have a genetic basis, which determines the potential immune response.

### 1.6.2 *In vitro* gene expression

Studies have examined the relationship between individual polymorphic alleles or haplotypes of cytokine genes and the expression of the cytokine *in vitro*. The majority have involved identifying cells with a particular allele and measuring the levels of cytokine or cytokine mRNA expressed as a result of *in vitro* stimulation with a mitogen. Results may be conflicting, depending on the cell lineage used and the treatment of subjects prior to harvesting cells. There is a large excess of known polymorphisms that have not been subject to *in vitro* expression studies (Bidwell 1999).

### 1.6.3 *In vivo* disease association

These studies attempt to identify immunogenetic markers for a given disease. Individual cytokine genotypes are compared with clinical features of a disease e.g. incidence, duration, severity and outcome. The polymorphisms studied are usually determined by a reported dysregulation of a particular cytokine or cytokine receptor in the disease. Many studies have identified statistically significant associations between cytokine alleles and disease. Many more have failed to do so partly because of the difficulty in selecting a polymorphism for study from amongst the many candidate possibilities and partly because of the likely modest effect of any single polymorphism within the highly complex cytokine network. A few studies have attempted to analyse the combined contribution of more than one cytokine gene polymorphism to disease. Despite the difficulties, the importance of such studies goes beyond improving the understanding of the immune response in human disease. They may ultimately identify markers of disease susceptibility, severity and clinical outcome and identify targets for intervention such as vaccines(Bidwell 1999).

### 1.6.4 Cytokines and the cervix

There is a higher rate of cervical disease in women who are immunosuppressed i.e. those with HIV, lymphomas or on post transplant immunosuppressive regimes. There are decreased Langerhans cell counts in normal epithelium in smokers and pregnant women. Local cervical immunity is suppressed by smoking and the COCP (Poppe 1996).

IL-1α, IL-1β, IL-1Ra and TNF-α are all produced by endo and ecto cervical epithelium in the normal cervix.

In an earlier study Majeed et al identified an association between the high secretor phenotype of IL-1β and CIN (Majeed 1999).

The highly significant nature of this association prompted investigation of other related cytokine gene polymorphisms.

### 1.6.5 IL1 Receptor antagonist (IL-1Ra)

The IL-1Ra gene lies in the IL-1 cluster on chromosome 2. IL-1Ra is structurally related to IL-1β (26% homology) and it competes for occupancy of IL-1 surface receptors. It acts as a regulator for IL-1α and IL-1β by inhibiting their release (Hurme 1998). It also binds competitively to the IL-1 receptor, blocking binding of IL-1α and IL-1β. IL-1Ra is detectable in the circulation of normal healthy people unlike IL-1α and IL-1β (Hurme 1998). There is a polymorphism in intron 2 of the IL-1Ra gene caused by the variable copy number of an 86-bp sequence (Tarlow 1993)( Table 1.5). Five alleles represent 240-, 325-, 410-, 500-, and 595-bp copies of the repeat sequence. The rare allele 2 is associated with increased levels of IL-1Ra secretion (Hurme and Santtila 1998). Allele 2 is therefore associated with reduced IL-1α production (Danis 1995). Allele 2 has been linked with inflammatory epithelial disease (Tarlow 1994) and with increased susceptibility to vulval vestibulitis (Jeremias 2000). It has also been associated with systemic lupus erythematous, ulcerative colitis, psoriasis, alopecia areata and Graves disease (Bioque 1995, Blakemore 1994, Blakemore 1995, Tarlow 1997). The relative levels of IL-1Ra and IL-1 at an inflammatory site determine whether a pro-inflammatory response will be initiated and persist or will be terminated. Typically the concentration of IL-1Ra increases late in the course of the inflammatory event so that an induced acute inflammation can terminate and does not become chronic, damaging healthy cells (Sehouli 2002).

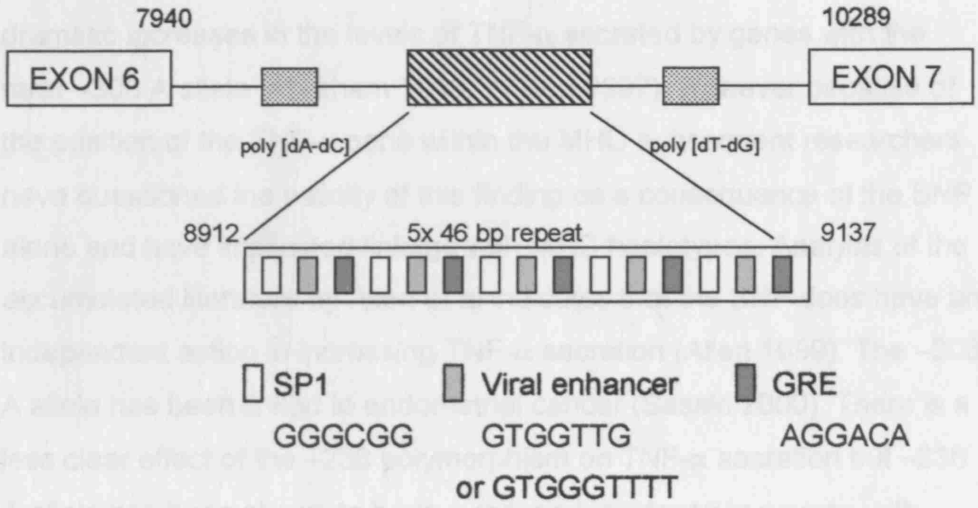
*Table 1.5* IL-1Ra gene polymorphism in intron 2

| Allele | No. of copies of 86 bp repeat | Characteristics | Frequency |
|---|---|---|---|
| 1 | 4 | Wild-type | 0.72 |
| 2 | 2 | Prolonged inflammation | 0.21 |
| 3 | 5 | Rare | 0.01 |
| 4 | 3 | Rare | 0.007 |
| 5 | 6 | Rare | 0.007 |

### 1.6.6 IL-1α

The IL-1α gene is also in the IL-1 cluster on chromosome 2. It shows 20% sequence homology with IL-1β. It is known that intron 6 of IL-1α shows a 5 x 46 bp tandem repeat in the genomic sequence. Sequencing demonstrates that the polymorphism is due to a variable number of repeats of the 46 bp sequence. 6 alleles ranging from 5 to 18 repeats have been identified, the most frequent allele (62%) containing 9 repeats (Bailly 1993, Bailly 1993). Each repeat contains three potential binding sites for transcriptional factors; a recognition sequence for the transcription factor SP1, a viral enhancer element and a glucocorticoid responsive element.

*Figure 1.5* Intron 6 of IL-1α




It has not been possible to demonstrate a statistically significant relation between in vitro IL-1α production and the number of repeats although there was a clear trend towards an inverse relation (Bailly 1996). Increased levels of IL-1α are associated with growth of abnormal cervical cells (Woodworth 1995).

### 1.6.7 TNF-α

TNF-α is a potent pro-inflammatory cytokine. It has direct cytolytic and cytostatic effects on tumours and in addition it stimulates production of IL-1 and MHC class I expression. TNF-α activates macrophages to tumouricidal activity both *in vitro* and *in vivo* (Talmadge 1987). TNF-α levels are reduced in women with breast, cervical and endometrial carcinoma and the level of reduction corresponds to increase in tumour size (De Jaco 1991).

The gene for TNF-α lies on the short arm of chromosome 6 which also contains major histocompatibility complex (MHC) genes. The coding region spans approximately 3kb and includes four exons and three introns. The capacity of monocytes to produce TNF-α varies more than ten fold. Single nucleotide polymorphisms (SNP) occur in the TNF-α gene at position –308 (G to A) and –238 (G to A). Initial research showed dramatic increases in the levels of TNF-α secreted by genes with the rarer –308 A allele (Abraham 1999, Wilson 1997). However because of the position of the TNF-α gene within the MHC subsequent researchers have questioned the validity of this finding as a consequence of the SNP alone and have implicated linkage with MHC haplotypes. Analysis of the accumulated literature by Allen et al indicates that the SNP does have an independent action in increasing TNF-α secretion (Allen 1999). The –308 A allele has been linked to endometrial cancer (Sasaki 2000). There is a less clear effect of the –238 polymorphism on TNF-α secretion but –238 A allele has been shown to have a reduced incidence in women with cervical cancer compared to normal controls (Calhoun 2002, Jang 2001).

*Table 1.6* Studies of cytokine levels in relation to cervical disease

| Author Year | Disease | Cytokine examined | Conclusions |
|---|---|---|---|
| Woodworth 1993 | Normal cervical cells, HPV infected cervical cells, cervical cancer | IL-1α, IL-1β, IL-1Ra, ,IL-6, IL-8, TNFα, GM-CSF | Reduced lymphokine production in diseased cells |
| Fukuda 1993 | Persistent and regressive CIN | S-100 positive Langerhans cells, helper induced T cells | Reduced immune response in persistent CIN |
| Elsasser-Beile 1993 | Whole blood from patients with gynaecological cancers and normal controls | IFN-γ, IL-1α, IL-2, TNF-α | IFN-γ, IL-1α and IL-2 levels reduced in cancer |
| Kyo 1994 | HPV 16 gene expression study from HPVI cells | TNF-α, IL-1α, IL-6, IF-γ | TNF-α and IL-1α inhibit HPV 16 expression |
| Tsukui 1996 | Peripheral blood lymphocytes from patients with HPVI, LSIL, HSIL and cervical cancer | IL-2 | Reduced cytokine production with increased severity of disease |
| Clerici 1997 | Peripheral blood mononuclear cells from HPV infected patients with CIN | Th1; IL-2, IFN-γ, Th2; IL—4, IL-10 | Reduced IL-2 and increased IL4 and IL-10 in more extensive CIN |
| Fujiwaki 1997 | Serum from patients with gynaecological cancers and benign conditions | IL-1Ra | Increased IL-1Ra in women with cancer compared to benign conditions |

| | | | |
|---|---|---|---|
| Malejczyk 1997 | HPV associated benign and malignant anogenital lesions | sTNF-R types I & II | Normal levels in CIN I-III, Increased levels in cervical cancer |
| Sheu 1997 | Cervical cancer | sTNF-RI (soluble TNF receptor) | Reduced levels in cervical cancer |
| Jacobs 1998 | Whole blood from patients with LSIL, HSIL and normal controls | Th1;IL-12, IFN-γ Th2; IL-4, IL-10 | Lower IL-12/IL-10 ratio in cervical disease |
| Scott 1999 | Cervical mucus from HPV infected patients | Th1 cytokines | Th1 response preceded clearance of HPV |
| Tjiong 2001 | Cervicovaginal washings from normal controls, CIN and cervical cancer | IL-1β, TNF-α, IL-12p40, IL-10, TGF-β1 | Increased cytokine levels in disease compared to controls |
| Fujiwaki 2003 | Tissue and serum from patients with cervical cancer and normal controls | IL-1Ra | Increased IL-1RA in squamous cell carcinoma but not adenocarcinoma |

(Clerici 1997, Elsasser-Beile 1993, Fujiwaki 1997, Fujiwaki 2003, Fukuda 1993, Jacobs 1998, Kyo 1994, Malejczyk 1997, Scott 1999, Sheu 1997, Tjiong 2001, Tsukui 1996, Woodworth 1993)

These studies have focused on the levels of cytokine present in the tissue or serum of patients and controls. This is subject to confounding factors such as the presence of intercurrent disease at the time of sampling. The advantage of examining cytokine gene polymorphisms is that the inherent ability of the patient to produce a particular cytokine is not subject to

these confounding factors. The influence of environmental factors such as smoking must still be considered.

### 1.6.8 Extended Haplotype analysis

As previously stated, single cytokine gene polymorphisms may have an effect on disease process in isolation but the complexity and pleiomorphic nature of the cytokine network means that a combination of polymorphisms is more likely to exert a significant effect. The construction of haplotypes is possible experimentally, through genotyping of additional family members or by statistical method. Several statistical programmes are available (Terwilliger 1992). The phase reconstruction method developed by Stephens et al has the advantage of increased accuracy but also estimates the uncertainty in each phase call. This avoids inappropriate overconfidence in statistically reconstructed haplotypes (Stephens 2001).

## 1.7 Hypothesis

The subsequent research has explored the hypothesis that polymorphisms of these cytokines might compromise the normal cervical response to HPV, such that these patients have either persistent HPV carriage, or are more likely to carry high-risk HPV. Furthermore such compromise might increase the risk that low-grade changes will progress to high-grade and/or cancer, and not regress as the majority will do.
As previously discussed there is no single screening tool with adequate sensitivity and specificity to offer definitive guidance to the management of women with smears suggesting low grade disease. At the time of this study risk models were being postulated including cytology, HPV testing and constitutional factors such as smoking. The models might be strengthened by the inclusion of further robust markers for disease outcome. The aim of the study was to identify such markers within the immune response to HPVI.

## METHODS

### 2.1 Patient recruitment

Patients were recruited from the colposcopy clinic at St Michael's Hospital, Bristol over a three year period from 1997 to 2000. The clinic receives referrals from the Avon Health Authority Cervical Cytology Screening Programme.

Low grade group; Patients (n=209) with smears showing mild dyskaryosis on two occasions six months apart and those showing borderline changes on three occasions six months apart formed the main study group.

High grade group; Patients (n=67) with smears showing moderate or severe dyskaryosis on one occasion who were referred to the clinic and whose subsequent histology confirmed a diagnosis of HSIL formed a further group.

Controls (n=47) were recruited from the Family Planning clinic at the Central Health Clinic, Bristol. They gave a history of compliance with the NHSCCP guidelines and consistently negative smears.

All patients were given written information about the study and written consent was obtained (Appendix 2). A full medical, sexual and family history was recorded on a standard form. Patients were excluded if they had any history of previous cervical treatment or if colposcopy was unsatisfactory i.e. if the TZ was not completely visualised. All patients were assigned a unique identifier code with which all samples were labelled to preserve confidentiality.

The study was approved by the United Bristol Healthcare Trust (UBHT) Ethics Committee.

### 2.2 Cervical smears

Cervical smears were taken at the recruitment visit on all women in the low-grade group. The smears were taken with an Aylesbury spatula and marked for the attention of a single cytologist.

### 2.3 HPV cervical sampling

Following the cervical smear two cervical brushes (Cervex Brush; International Medical Products, Zutphen, The Netherlands) were used to

acquire cervical scrapes; the first from the transformation zone in a 360° movement and the second over the remainder of the ectocervix. The brush heads were placed in 5ml sterile phosphate buffered saline (PBS) with 0.05% merthiolate in a Sterlin Universal tube. The samples were stored at –20 °C until DNA extraction.

### 2.4 Colposcopy

All patients in the low-grade group underwent colposcopy examination. The SCJ was identified. Three percent acetic acid was applied and the cervix examined at four, six and ten times magnification. Lugol's iodine (5% iodine and 10% potassium chloride in water) was used for Schiller's test. At each stage the cervix was imaged using Denvu 3100 Medical Imaging System (Denvu Inc., Tuscon, Arizona, USA). The images were stored on Syquest optical discs.

### 2.5 Cervical biopsies

All the patients in the low-grade group had colposcopic guided punch biopsies taken of the area of the TZ with the most abnormal appearance with Eppendorfer/Tischler Morgan biopsy forceps according to NHSCSP guidelines (Luesley 1996). These were sent for histological examination and marked for the attention of a single histopathologist. Some biopsies were divided in half and half was snap frozen for future research.
All patients in the high-grade group had a LLETZ biopsy as a treatment procedure and the samples were sent for histlological examination.

### 2.6 Blood Samples

All patients in both groups and all controls had 20-30ml of blood taken by standard venepuncture in EDTA bottles. These were stored at 4 °C until DNA extraction.

### 2.7 Follow Up

The high-grade group was managed according to local colposcopy clinic guidelines appropriate to their histology result.
The low-grade group was followed up according to their cytology, colposcopy and histology results. Any patient with entirely normal results was reassured by letter and discharged from the clinic. Patients with evidence of HSIL were recalled and offered treatment by LLETZ. The

further follow up of these patients was based on clinical need and did not form part of the study.

The remaining patients with low-grade disease were seen in colposcopy clinic at six and 12 months for a further smear, HPV scrape, and colposcopy examination. Further images were obtained. Punch biopsies were carried out if clinically indicated.

### 2.8 DNA Extraction by salting out method

The DNA extraction technique originally described by Miller (Miller 1988) is rapid and avoids organic extraction of proteins.

20ml of blood was collected in EDTA anticoagulant vacutainer tubes. It was transferred to a conical tube and centrifuged at 1300 x *g* for 10 minutes.

The buffy layer (approximately 1ml) was transferred to a 10ml disposable plastic conical tube. 8ml of red cell lysis buffer (0.144 *M* ammonium chloride, 1 m*M* sodium hydrogen carbonate) was added, mixed and the tubes allowed to stand at room temperature for 20 minutes.

The tubes were centrifuged at 1300 x *g* for 10 minutes. The red cell lysate was removed as near to the white cell pellet as possible. The white cell pellet was resuspended in 3ml nuclei lysis buffer (10 m*M* Tris-HCl pH 8.2, 0.4 *M* NaCl, 2 m*M* EDTA pH 8.0). 0.6ml 1x Proteinase K solution (2 mg/ml protease K in 1% w/v sodium dodecyl sulfate (SDS), 2 m*M* EDTA pH 8.0) and 0.2ml 10% w/v SDS was added.

The tubes were mixed and incubated for 18 hours at 37°C.

1ml 6 m*M* NaCl was added and the tubes shaken vigorously for 15 seconds.

The tubes were centrifuged for 25 minutes at 1300 to 1500 x *g*.

The supernant was pipetted into a clean disposable plastic tube, avoiding the pellet. 8ml absolute ethanol was added and the solution mixed by gentle inversion.

The precipitated DNA was removed with a sealed glass Pasteur pipette and the excess ethanol allowed to evaporate. The DNA was redisolved in 0.1 to 0.3ml distilled water.

The concentration of DNA was calculated by adding 10μl of the DNA solution to 990μl distilled water and measuring the optical density at 260nm. The concentration of DNA (in μg/μl) is 5 x the optical density. The DNA was stored at -20°C until required.

**2.9 Cytokine gene polymorphism analysis**

These analyses were carried out according to the previously published methods.

1. TNFα -308 and –238 (Morse 1999)
2. IL-1α (Bailly, di Giovine 1993)
3. IL-1Ra (Tarlow, Blakemore 1993)

Polymerase chain reaction primers

The primers used to amplify the DNA segments containing the polymorphism sites were synthesised in house on an Applied Biosystems oligonucleotide synthesiser.

Primers

1. TNFα - 308 polymorphism
   Forward: 5'-TCCTGCATCCTGTCTGGAAG-3'
   Reverse: 5'- GTCTTCTGGGCCACTGACTG-3'
2. TNFα - 238 polymorphism
   Forward: 5'- GTTCAGCCTCCAGGGTCCTACACA-3'
   Reverse: 5'- GGGATTTGGAAAGTTGGGGACACA-3'
3. IL-1α
   Forward: 5' –GTGAGGTCAGGCCATTGCAC-3'
   Reverse: 5'-CTCCACTGATTAAGTAGGTGACTC-3'
4. IL-1Ra
   Forward: 5'CTCAGCAACACTCCTAT-3'
   Reverse: 5'TCCTGGTCTGCAGGTAA-3'

PCR conditions

In each case negative controls were included at the start and end of each run in order to confirm that no contamination had occurred.

1. TNFα -308 and –238

PCR mixes (50μl) contained 0.5μM of each of forward and reverse primers, 2.5μM $MgCl_2$, 200μM of each dNTP, 1 x *Taq* polymerase buffer (75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% V/V Tween), 0.5 unit *Taq* polymerase (Advanced Biotechnologies) with 500 ng genomic DNA. PCR was carried out on a Stratagene Robocycler Gradient 96 thermal cycler by initial denaturation at 95°C for 5 minutes, 35 cycles of 95 °C for 30 seconds, 59 °C for 1 minute (58 °C for the –238 polymorphism), 72 °C for 30 seconds and final extension at 72 °C for 5 minutes.

2. IL-1α

PCR mix was as for TNFα polymorphisms. PCR parameters were initial denaturation at 95°C for 5 minutes, 35 cycles of 95 °C for 30 seconds, 55 °C for 1 minute, 72 °C for 1 minute and final extension at 72 °C for 5 minutes.

3. IL-1Ra

PCR mix was as for TNFα polymorphisms. PCR parameters were initial denaturation at 95°C for 5 minutes, 35 cycles of 95 °C for 30 seconds, 52 °C for 1 minute, 72 °C for 1 minute and final extension at 72 °C for 5 minutes.

### 2.10 Induced heteroduplex generators (IHGs)

IHGs were constructed for the TNFα polymorphisms as single long oligonucleotides using 0.2μm membrane columns on a PerSeptive Expedite 8900 Nucleic Acid Synthesis System. These IHGs are identical in sequence to the target gene except they have been deliberately modified immediately adjacent to the polymorphic loci, by the inclusion of a 4 base adenine insert either upstream (TNFα - 308) or downstream (TNFα -238) of the polymorphic nucleotide (Fig 2.1). Following synthesis, deprotection and precipitation, the oligonucleotides were amplified by PCR using the appropriate primers.

Equal volumes (5-10μl) of amplicons from genomic DNA and IHGs were mixed, denatured at 95 °C for 5 minutes and allowed to cool slowly from 95 °C to 37 °C over a 30 minute period in a Biometra UNO II. This generates heteroduplexes with different conformational forms and

therefore different electrophoretic mobilities which are characteristic for individual alleles.

*Figure 2.1* Induced heteroduplex generators

Heteroduplex generator for TNFα - 308 G to A transition

Allele 1 TCCTGCATCCTGTCTGGAAAGTTAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAGAAATGGAGGCAATAGGTTTTGAGGG

Allele 2 ----------

HG ----------

Allele 1 GCATTG GGGACGGGGTTCAGCCTCCAAGGGTCCTACACACAAATCAGTCAGTGGCCCAGAAGAC

Allele 2 -------- A----------

HG ---------GGGG----------

Heteroduplex generator for TNFα - 238 G to A transition

Allele 1 GTTCAGCCTCCAGGGTCCTACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTCAGAATCG GAGCAGGGAGGATGGG

Allele 2 ----------A ----------

HG ---------- AAAA----------

Allele 1 GAGGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAAATCCC

Allele 2 ----------

HG ----------

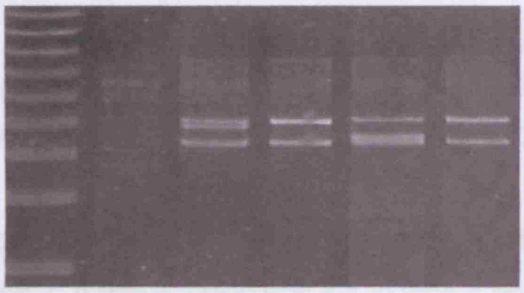
## 2.11 Non denaturing polyacrylamide minigel electrophoresis

PCR products were resolved on non-denaturing polyacrylamide "triple wide" minigels (30cmx8cm: CBS Scientific Company, Del Mar, USA) in a Bio-Rad Mini-Protean II gel electrophoresis system at 200v.

1. TNFα -308 and –238

The heteroduplexes were resolved on 15% Protogel (National Diagnostics) for 105 minutes. The gels were stained for 5 minutes with 1 x TBE containing 0.5μg/ml ethidium bromide. The gels were examined using a 302nm UV transilluminator and recorded via a Glyko FACE digitising CCD imager.

*Figure 2.2* Band pattern for TNFα -308 and –238 genotyping showing a marker lane, negative control lane, genotype -308 GG and -238 GG in lanes 4 and 6, genotype -308 GA and -238 GG in lane 3 and genotype –308 GG and –238 GA in lane 5.



| | | | | | |
|---|---|---|---|---|---|
| -308 | | GA | GG | GG | GG |
| -238 | | GG | GG | AG | GG |

2. IL-1α

The PCR products were resolved on 12% Protogel (National Diagnostics) for 65 minutes. The gels were stained with silver stain and viewed as above. The silver stain method used was published by Merril et al (Merril 1984). The gel was fixed for four hours at room temperature with gentle shaking in a solution of ethanol:glacial acetic acid:water (30:10:60). It was then incubated in 30% ethanol for 30 minutes at room temperature. This step was repeated with fresh ethanol. The gel was incubated for 10 minutes in fresh deionised water three times. The gel was incubated for 30 minutes in 0.1% $AgNO_3$. It was washed with deionised water and incubated in freshly made aqueous 2.5%

sodium carbonate, 0.02% formaldehyde. The banding pattern appeared over a few minutes. The gel was washed in 1% acetic acid to quench the reaction and then washed several times in deionised water (10 minutes per wash).

*Figure 2.3* Band pattern for IL-1α showing marker and negative control in lanes 1 and 2, genotype 9,9 in lane 3, genotype 8,8 in lane 5 and genotype 8,9 in lanes 4 and 6.

3. IL-1Ra

The PCR product were resolved on 12% Protogel (National Diagnostics) for 55 minutes. They were stained and viewed as for TNFα -308 and –238.

*Figure 2.4* Band pattern for IL-1Ra showing genotype 1,3 in lanes 1 and 2, genotype 1,2 in lanes 3, 5 and 6, genotype 1,1 in lane 4 and genotype 1,4 in lane 7.


**2.12 Sample preparation of cervical smears for HPV typing.**

Smears were taken with a cervix brush from the transformation zone and stored in 5ml PBS (10mM phosphate, 150mM NaCl, pH 7.2) with 0.05% Merthiolate.

The brushes were vortexed vigorously and the cervix brushes were removed with sterile forceps

The cells were pelleted by centrifugation at 3000 x *g* for 10 minutes and the supernant carefully decanted.

1ml of 10mM Tris-HCl, pH 7.4 was added to the cell pellet using a disposable pipette and gently vortexed before transferring the whole cell suspension (1ml) to a new tube.

The cell suspensions was frozen at –70°C and then thawed and boiled at 100 °C for 10 minutes in a waterbath, then cooled on ice.

The tubes were centrifuged for 1 minute at 3000 x *g*.

The tubes were vortexed prior to use.

During the sample preparation, two empty tubes (the first and last tubes) were processed along with the samples. At the end of sample preparation, these first and last tubes should contain 10µl of 10mM Tris-HCl and served as negative controls.

### 2.13 HPV typing

The samples were HPV typed in the Molecular Pathology laboratory of the Vrije Universiteit, Amsterdam under the supervision of Rene Pol (Chief Technician in the molecular biology group) according to the published method by van den Brule (van den Brule 1990).

### 2.14 β-Globin PCR amplification for DNA quality control.

Because the HPV detection was performed on crude cell suspensions rather than extracted DNA, this procedure acted as a quality control measure to ensure cellular DNA was present in the samples and to reduce the possibility of false negative results.

10µl of cervical cell suspension was added to a PCR mix containing 200µM dNTPs, 1x PCR buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 25pmol forward and reverse primers, 1U AmpliTaq DNA polymerase (Perkin Elmer) made up to 40µl with sterile water.

The primers used were

Forward: 5'-ACACAACTGTGTGTTCACTAGC-3'

Reverse: 5'-GAAACCCAAGAGTCTTCTCT-3'

The PCR parameters were 4 minutes at 94°C, 40 cycles of 1 minute at 94 °C, 2 minutes at 55 °C, 90 seconds at 72 °C, and finally 4 minutes at 72 °C.

### 2.15 HPV GP5+/bio6+ PCR amplification procedure

10µl of crude cervical cell suspension was added to a PCR mix containing 200µM dNTPs, 1x PCR buffer II (Perkin Elmer), 3.5 mM $MgCl_2$, 25pmol forward and reverse primers, 1U AmpliTaq DNA polymerase (Perkin Elmer) made up to 40µl with sterile water.

The primers used were

GP5+: 5'-TTTGTTACTGTGGTAGATACTAC-3'

bioGP6+: 5'-GAAAAATAAACTGTAAATCATATTC-3'

The PCR parameters were 4 minutes at 94°C, 40 cycles of 1 minute at 94 °C, 2 minutes at 40 °C, 90 seconds at 72 °C, and finally 4 minutes at 72 °C.

### 2.16 Enzyme Immunoassay detection of PCR products

The PCR products of both the β-globin PCR and the HPV GP5+/bioGP6+ PCR were detected by EIA.

5µl of PCR product was pipetted into a streptavadin coated microwell (Boehringer-Mannheim High capacity) according to a predetermined plate configuration. 50µl of fresh 1x SSC/0.5% Tween 20 solution (0.15 M NaCl, 0.015 M NaCitrate, Tween 20 Merck) was added to each well. The plate was covered and incubated at 37°C for 60 minutes.

The microwells were washed 3 times with 1x SSC/0.5% Tween 20 using an automated plate washer and excess liquid was tapped out. 100µl of 0.2M NaOH was added to each well. The plate was covered and incubated at room temperature for 15 minutes.

The microwells were washed again as above. 50µl of probe mixture (a mixture of digoxigenin-11-ddUTP labelled oligonucleotides specific for the HPV types under study) was added to each well. The plate was covered and incubated at 37 °C for 60 minutes.

The microwells were washed as above. 50µl of conjugate (dilute alkaline phosphatase conjugated to anti-digoxigenin-11-ddUTP Fab fragments (Boehringer-Mannheim; 0.75 U/µl) 1:10.000 in 1x SSC/0.5% Tween 20 to a final concentration of 0.75mU/ml) was added to each well. The plate was covered and incubated at 37 °C for 60 minutes.

The wells were washed 5 x as above. 100µl substrate (1mg pNPP/ml 0.2 M Tris-HCl (Sigma)) was added to each well. The plate was covered and incubated at 37 °C for 60 minutes.

The absorbance was read with an EIA reader at 405nm using a reference filter at 620nm. The plate was covered, sealed in a plastic bag and incubated overnight at 37 °C.

The optical density (OD) was read with an EIA reader at 405nm using a reference filter at 620nm.

Negative controls were included during PCR and EIA stages to avoid false positive results due to contamination. A stock of positive controls (PCR products derived cloned HPV 16) were used as a reference standard.

**2.17 Statistical analysis**

Power calculations were carried out prior to the study. The calculations were based on demonstrating an odds ratio of 3.0 with 95% confidence intervals and 80% power.

For TNFα-308 the genotype frequencies used for the calculation were G 84% and A 16% giving phenotype frequencies of 0.974 and 0.294 based on phenotype frequency = 1-(1-genotype frequency)$^2$. This resulted in a sample size of 94 patients and 47 controls (Hamann 1995).

For TNFα-238 the genotype frequencies were G 91% and A 9% giving phenotype frequencies of 0.992 and 0.172. This resulted in a sample size of 120 patients and 60 controls (Hamann, Mantzoros 1995).

For IL-1α the allele frequencies used were A1 61.8%, A2 22.2%, A3 7.6%, A4 4.9%, A5 2.1% and A6 0.7%. This resulted in a sample size of 113 patients and 56 controls(Bailly, di Giovine 1993).

For IL-1Ra the allele frequencies used were allele1 72%, allele 2 21%, allele 3 1%, allele 4 0.7% and allele 5 0.7%. This resulted in a sample size of 140 patients and 70 controls. (Tarlow, Blakemore 1993).

The age of patients in the various groups was analysed using Kruskal-Wallis test as a one way analysis of variance in a non parametric dataset.

The parity and contraceptive use of patients were analysed using Fisher-Freeman-Halton exact test. Incomplete data were excluded. Smoking behaviour was analysed using Yates corrected $\chi^2$ test. The Yates correction

allows for integer entered values in a continuous variable. Incomplete data were excluded.

The disease outcome in relation to cervical disease and smoking behaviour were analysed using $\chi^2$ or Fisher's exact as some fields contained low numbers of patients. The patients lost to follow up were excluded.

The HPV infection in patients with low grade smears and their disease outcome was analysed using $\chi^2$ test with Fisher's exact where fields contain low numbers. Patients without results and those lost to follow up were excluded.

The cytokine gene polymorphism results were analysed using $\chi^2$ test with Fisher's exact where fields contain low numbers. Because of the multiple tests performed, Bonferroni's correction was applied. This technique divides the test-wise significance level by the number of tests performed in order to reduce the chance of a type I error. This technique has the drawback of increasing the chance of a type II error. Missing results were once again excluded. There is a risk of excluding missing results introducing bias.

The statistical analysis was performed using Statcalc (EpiInfo 6 1993, Centre for Disease Control USA) and Stats Direct (2006). The haplotype analysis was performed using Phase software (Stephens, Smith 2001).

## RESULTS

### 3.1 Patient Characteristics

#### 3.1.1 Age

The age range for the patients recruited to the study is shown in the Table 3.1. The group includes patients who, at 18 and 19 years old, are below the recommended age for cervical screening. Their smears were taken opportunistically based on symptoms and history of early first intercourse. They were then referred to colposcopy clinic after abnormal smear results according to the same protocols as women in the recommended screening ages.

*Table 3.1.* Age distribution of recruited patients

| | Normal control n=47 | Low grade smear n=209 | HSIL n=67 |
|---|---|---|---|
| Minimum age | 20 | 18 | 21 |
| Maximum age | 43 | 62 | 49 |
| Average age | 30.44 | 31.50 | 32.81 |
| Median age | 30 | 29 | 30 |

There is no significant difference in the age distribution between normal controls and patients with low grade smears or HSIL (p=0.229 Kruskal-Wallis test 95% CI 2df)

#### 3.1.2 Parity

There is a trend to increasing parity with increased severity of cervical disease. Eighty one per cent of normal controls were nulliparous compared to 43% of patients with low grade smears and 19% of patients with HSIL. By contrast only eight per cent of normal controls had two or more children while 33% of those with low grade smears and 42% of those with HSIL did. The women with abnormal cytology were significantly more likely to be parous than the normal controls (p=0.0001 Fisher-Freeman-Halton exact 95% CI df8, incomplete data excluded) (Table 3.2).

*Table 3.2.* Parity of recruited patients.

| Parity | Normal control | Low grade smears | HSIL |
|---|---|---|---|
| 0 | 38 (81%) | 90 (43%) | 13 (19%) |
| 1 | 5 (11%) | 43 (20%) | 7 (10%) |
| 2 | 4 (8%) | 46 (22%) | 18 (27%) |
| 3 | 0 | 18 (9%) | 5 (8%) |
| >4 | 0 | 4 (2%) | 5 (8%) |
| Incomplete data | 0 | 8 (4%) | 19 (28%) |

### 3.1.3 Contraception

Table 3.3 shows the contraceptive use of the recruited patients. There was no difference between the groups in terms of use of contraception compared to use of no contraception (p=0.108 Fisher-Freeman-Halton exact 95% CI df10, incomplete data excluded). The most commonly used method was the combined oral contraceptive pill used by 43% of controls, 44% of patients with low grade smears and 42% of patients with HSIL. Fifteen per cent of normal controls used an IUCD compared to 10% of patients with low grade smears and nine per cent of patients with HSIL. The use of barrier contraception was most common in the low grade smear group (11%) compared to four per cent in the control group and three per cent in the HSIL group however this did not reach statistical significance. (p=0.0638 Fisher-Freeman-Halton exact 95% CI df10, incomplete data excluded). There were no sterilised women in the control group as these women were recruited at a family planning clinic and were therefore actively seeking contraceptive advice or services.

*Table 3.3.* The contraceptive use amongst recruited patients

| Form of contraception | Normal control | Low grade smears | HSIL |
|---|---|---|---|
| None | 13 (28%) | 36 (17%) | 18 (27%) |
| Barrier | 2 (4%) | 23 (11%) | 2 (3%) |
| OCP | 20 (43%) | 91 (44%) | 28 (42%) |
| Depo Provera | 5 (10%) | 9 (4%) | 2 (3%) |
| IUCD | 7 (15%) | 20 (10%) | 6 (9%) |
| Sterilisation | 0 | 22 (11%) | 7 (10%) |
| Incomplete data | 0 | 8 (3%) | 4 (6%) |

### 3.1.4 Smoking

Smoking was more common amongst patients than controls, with 21% of controls being smokers compared to 44% of women with low grade smears (p=0.005 Yates corrected $\chi^2$ test 95% CI)(Table 3.4).

*Table 3.4.* The smoking behaviour of recruited patients

| | Normal control | Low grade smears |
|---|---|---|
| Smoker | 10 (21%) | 92 (44%) |
| Non smoker | 37 (79%) | 113 (54%) |
| Incomplete data | 0 | 4 (2%) |

## 3.2 Patients referred with low grade smears

### 3.2.1 Disease at recruitment amongst patients with low grade smears

Two hundred and nine patients referred with low grade smears were assessed cytologically (with a further smear), colposcopically and histologically (with a directed punch biopsy). Forty seven (22%) of patients had a normal smear, biopsy and colposcopy and were discharged from the clinic. Thirty three (16%) had a smear or punch biopsy suggesting a high grade lesion and were treated with LLETZ. In all but four cases LLETZ histology confirmed high grade disease. In two cases LLETZ histology showed CIN I, following a punch biopsy showing CIN II. In two cases cytology showed moderate dyskaryosis and the patients were offered treatment. One of these patients defaulted to any further appointments and for the other LLETZ histology showed no CIN following a punch biopsy showing CIN I. In all cases where colposcopy was suspicious of a high grade lesion this was supported by cytology and/or histology. In all 80 (38%) patients in the above categories were excluded from the study.

The remaining 129 patients (62%) with evidence of low grade disease were followed up. Twenty three (11%) of patients had CIN I on histology. The remaining 106 patients had no evidence of CIN on histology. A further 35 patients had a smear showing mild dyskaryosis. These patients were classified with the CIN I group as mild dyskaryosis/CIN I (n=58 28%). Seventy one patients (34%) had a smear showing borderline change or a biopsy showing koilocytosis (23 (11%) borderline and 48 (23%) koilocytes)

with no more significant abnormality. These patients were classified together as borderline change/koilocytosis.

Where histology and cytology disagreed the more abnormal diagnosis was used i.e. in a patient with CIN I and a borderline smear, the diagnosis was CIN I. The cytology and histology agreed in 51 (40%) patients. In 32 (25%) patients histology was abnormal while cytology was normal and in 46 (35%) the histology was normal and the cytology abnormal.

*Table 3.5* Cytology and histology at recruitment in patients with low grade smears shown to have low grade disease

| Histology | Cytology | | | |
|---|---|---|---|---|
| | Normal | Mild dyskaryosis | Borderline | Total |
| CIN I | 5 | 14 | 4 | 23 |
| Koilocytosis | 27 | 12 | 21 | 60 |
| Inflammation | | 11 | 11 | 22 |
| Metaplasia | | 1 | 1 | 2 |
| Normal | | 11 | 11 | 22 |
| Total | 32 | 49 | 48 | 129 |

### 3.2.2 Disease outcome in patients with low grade disease

Of the 129 patients with low grade disease, 25 (19%) were lost to follow up (failed to attend three invitations to colposcopy or moved to another area) and two requested treatment during the study and were therefore withdrawn. One hundred and two patients were followed up for 12 months.

Eleven (8%) patients progressed to HSIL based on histology, colposcopy and cytology and were treated with LLETZ. Nine of these progressed within six months. In all cases where colposcopy was suspicious of a high grade lesion this was supported by cytology and histology. In all cases LLETZ histology confirmed HSIL. Seven (64%) of these 11 patients had CIN I at recruitment and one had mild dyskaryosis and koilocytosis.

Nineteen (15%) of patients had persistent low grade disease at 12 months on cytology, colposcopy and histology. Patients with CIN I at presentation were

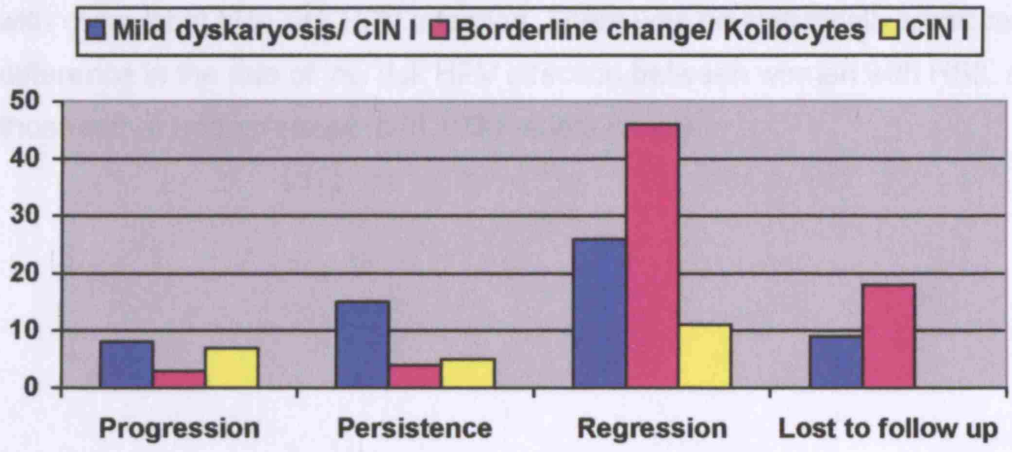
more likely to have disease progression or persistence than those without CIN I i.e. with koilocytic change or abnormal cytology unconfirmed by histology (p=0.0029 Fisher's exact).

Seventy two (56%) of patients experienced disease regression to normal cytology and colposcopy. Twenty six of these were within the first six months. Histology was only repeated in these patients if clinically indicated. Patients with borderline smears or koilocytosis were more likely to experience disease regression than those with mild dyskaryosis or CIN I (p=0.0001 Fisher's exact).

*Table 3.6* Disease outcome at 12 months for patients with low grade disease at recruitment

| Disease outcome | | Cervical disease | | | |
|---|---|---|---|---|---|
| | Total | CIN I | No CIN I | Mild dyskaryosis/ CIN I | Borderline change/ Koilocytosis |
| Progression | 11 (8%) | 7 (30%) | 4 (4%) | 8 (14%) | 3 (4%) |
| Persistence | 19 (15%) | 5 (22%) | 14 (13%) | 15 (26%) | 4 (6%) |
| Regression | 72 (56%) | 11 (48%) | 61 (58%) | 26 (45%) | 46 (65%) |
| Lost to Follow up | 27 (21%) | 0 | 27 (25%) | 9 (15%) | 18 (25%) |

*Figure 3.1* Disease outcome at 12 months for patients with low grade disease



### 3.2.3 Effect of smoking on disease outcome

Patients were less likely to experience disease regression if they were smokers (p=0.0008 test Fisher's exact df2)(Table 3.7).

*Table 3.7* Disease outcome of patient with low grade disease according to smoking behaviour

| Disease outcome | Smoking Status | | |
|---|---|---|---|
| | Smoker | Non Smoker | Not stated |
| Progression | 9 (16%) | 1 (1%) | 1 |
| Persistence | 12 (21%) | 7 (10%) | 0 |
| Regression | 24 (42%) | 46 (67%) | 2 |
| Lost to follow up | 12 (21%) | 15 (22%) | 0 |

## 3.3 HPV Infection

### 3.3.1 HPV infection in patients with low grade smears

Table 3.8 shows the incidence of HPV infection in patients recruited with low grade cervical smears. No results were obtained in 17 (8%) patients, either because the HPV swab was omitted in error or because the β-globin analysis was negative indicating that the swab contained no DNA suitable for HPV testing. Only one (3%) of the 33 patients whose diagnosis at recruitment was HSIL was negative for HPV infection as compared to 24 (51%) of those with a normal cervix (p=0.000005 $\chi^2$ test). However 18 (39%) women with a normal cervix were positive for high risk HPV. In those women with low grade disease, 40 (56%) of those with borderline change/ koilocytes were high risk HPV positive compared to 42 (72%) of those with mild dyskaryosis/CIN I (p=0.05 $\chi^2$ test). Most (59%) of low risk HPV infection occurred in patients with concurrent high risk HPV infection. There was no statistically significant difference in the rate of low risk HPV infection between women with HSIL and those with a normal cervix (p=0.193 Fishers exact).

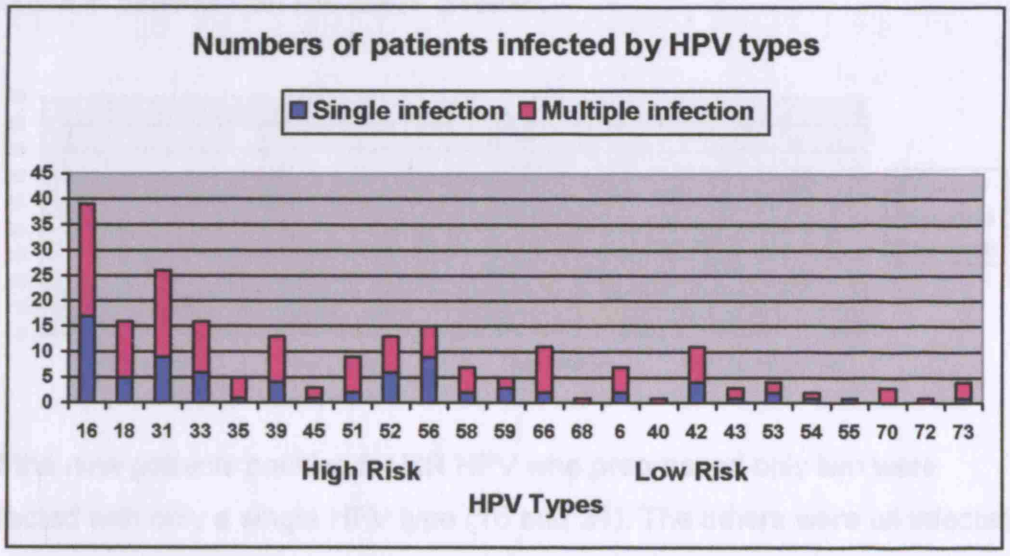
*Table 3.8* The incidence of HPV infection in women referred with low grade smears according to their disease at recruitment

| HPV status | Disease at recruitment | | | |
|---|---|---|---|---|
| | Normal cervix | Borderline change/ koilocytes | Mild dyskaryosis/ CIN I | HSIL |
| High risk HPV positive alone | 14 (30%) | 30 (42%) | 34 (58%) | 26 (79%) |
| Low risk HPV positive alone | 3 (6%) | 10 (14%) | 4 (7%) | 0 |
| Low risk and high risk HPV positive | 4 (9%) | 10 (14%) | 8 (14%) | 2 (6%) |
| HPV Negative | 24 (51%) | 15 (21%) | 7 (12%) | 1 (3%) |
| No result | 2 (4%) | 6 (9%) | 5 (9%) | 4 (12%) |
| Total | 47 | 71 | 58 | 33 |

### 3.3.2 HPV types in patients with low grade smears

Fig 3.2 shows the numbers of patients infected by each of the HPV types as part of a single or multiple infection in patients referred with low grade smears.

*Figure 3.2* HPV types in patients with low grade smears.

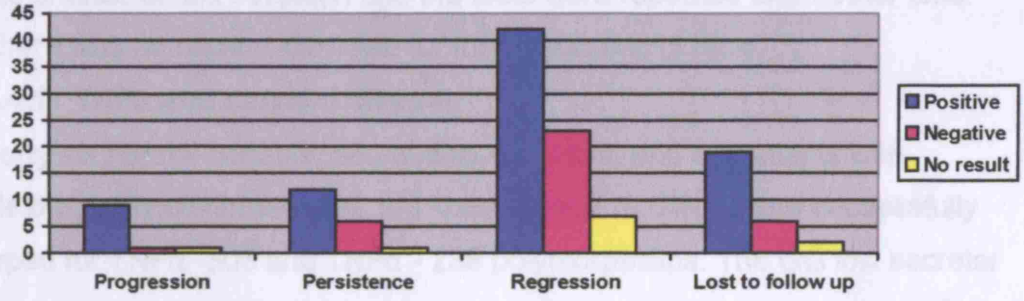


HPV 16 is the most common infecting type with 39 patients (31% of those with high risk HPV, 19% of the total) being positive for this type. The other commonly occurring types were 18 (13% of HR HPV positives), 31 (20%), 33 (13%), 39 (12%) and 52 (12%). Only 68 (54%) of the patients positive for HR HPV were infected with only one HPV type, the rest had infection with multiple HR HPV types. Eighty three (65%) patients positive for HR HPV types were positive for at least one of HPV 16, 18, 31, 33 and 35.

### 3.3.3 Disease outcome in women with low grade disease according to HPV infection

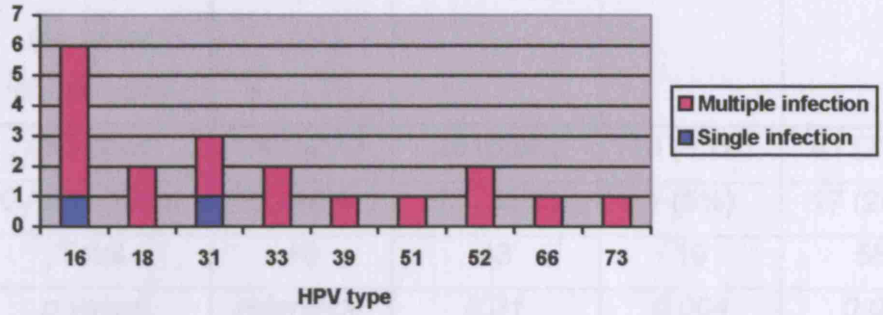
Examining the 102 women with low grade disease confirmed at recruitment and followed up for 12 months, all but two of the 11 patients who experienced disease progression were positive for high risk HPV. One was infected with HPV 70 and one had no HPV result. Thus patients who progressed were more likely to be infected with HR HPV (p=0.05 Fisher's exact test). However patients who were positive for high risk HPV were not statistically significantly more likely to progress than those negative for HPV (p=0.193 $\chi^2$ test Fishers exact). Moreover 42 (58%) patients who regressed were positive for high risk HPV (Fig3.3).

*Figure 3.3* Incidence of high risk HPV infection and disease outcome at 12 months in patients with low grade disease.




Of the nine patients positive for HR HPV who progressed only two were infected with only a single HPV type (16 and 31). The others were all infected with multiple HPV types.

*Figure 3.4* HPV types and incidence of disease progression




Testing for HPV 16 and 18 only would miss four (36%) of patients who experienced disease progression. Testing for HPV 16,18,31,33 and 35 would miss two (18%) patients.

## 3.4 Cytokine gene polymorphism results.

For the cytokine gene polymorphism analysis, only women with a histological diagnosis were included – those whose cytology and/or colposcopy was abnormal but not confirmed by histology were excluded. Not all gene polymorphism tests were successful. The expected drop out rate for these tests is five per cent. Unsuccessful tests were repeated twice and also repeated by another analyst. DNA from these patients was cleaned to remove the chance of salt inhibition and the tests were repeated one further time. There was no reason identified for the unsuccessful tests.

### 3.4.1 TNFα and cervical disease

Forty six normal controls, 58 patients with HSIL and 62 patients with histologically confirmed LSIL (43 koilocytosis, 19 CIN I) were successfully typed for TNFα -308 and TNFα - 238 polymorphisms. The GG low secretor phenotype of TNFα -308 is associated with all grades of CIN ($p=0.01$ $\chi^2$ test), the association being strongest with CIN I ($p=0.004$ $\chi^2$ test Fisher's exact). By contrast there was no association demonstrated with the koilocytes group ($p=0.31$ $\chi^2$ test). There was no association demonstrated between any of the cervical disease groups and the TNFα -238 polymorphism (Table 3.9).

*Table 3.9* TNFα -308 and –238 polymorphisms in women with cervical disease

| Polymorphism | Cervical diagnosis | | | | |
|---|---|---|---|---|---|
| TNFα-308 (secretor phenotype) | Normal | Koilocytes | CIN I | CIN II/III | All CIN |
| GG (low) | 24 (52%) | 28 (65%) | 18 (95%) | 41 (71%) | 59 (77%) |
| GA/AA (high) | 22 (48%) | 15 (35%) | 1 (5%) | 17 (29%) | 18 (23%) |
| Total | 46 | 43 | 19 | 58 | 77 |
| *p value* $\chi^2$ test | *reference group* | *0.31* | *0.004* | *0.09* | *0.01* |
| TNFα-238 | | | | | |
| GG | 41 (89%) | 38 (88%) | 17 (89%) | 50 (86%) | 67 (87%) |
| GA/AA | 5 (11%) | 5 (12%) | 2 (11%) | 8 (14%) | 10 (13%) |
| Total | 46 | 43 | 19 | 58 | 77 |
| *p value* $\chi^2$ test | *reference group* | *0.83* | *0.69* | *0.89* | *0.95* |

### 3.4.2 TNFα and disease outcome

With the number of patients studied there was no statistically significant association between the disease outcome for patients with low grade disease (koilocytes or CIN I) and their TNFα -308 (p=0.764 $\chi^2$ test Fisher's exact) or –238 (p=0.247 $\chi^2$ test Fisher's exact) phenotype (Table 3.10)

*Table 3.10* Disease outcome at 12 months related to TNFα -308 or –238 phenotype

| Disease outcome | TNFα -308 genotype | | TNFα -238 genotype | |
|---|---|---|---|---|
| | GA/AA | GG | GA/AA | GG |
| Progression | 2 (13%) | 5 (11%) | 1 (16%) | 6 (11%) |
| Persistence | 1 (6%) | 6 (13%) | 2 (28%) | 5 (9%) |
| Regression | 7 (43%) | 27 (59%) | 2 (28%) | 32 (58%) |
| Lost to follow up | 6 (38%) | 8 (17%) | 2 (28%) | 12 (22%) |
| Total | 16 | 46 | 7 | 55 |

### 3.4.3 TNFα and HPV infection

There is also no statistically significant association between high risk HPV infection and TNFα -308 (p=0.862 Fisher's exact test) or –238 (p=0.06 Fisher's exact test) phenotype (Table 3.11)

*Table 3.11* TNFα -308 and –238 genotype and high risk HPV infection

| High risk HPV | TNFα -308 genotype | | TNFα -238 genotype | |
|---|---|---|---|---|
| | GA/AA | GG | GA/AA | GG |
| Positive | 11 (69%) | 31 (67%) | 3 (43%) | 39 (71%) |
| Negative | 4 (25%) | 10 (22%) | 4 (57%) | 10 (18%) |
| No Result | 1 (6%) | 5 (11%) | 0 | 6 (11%) |
| Total | 16 | 46 | 7 | 55 |

### 3.4.4 IL-1α

Twenty seven normal controls, 47 patients with HSIL and 38 patients with histologically confirmed LSIL (14 CIN I, 24 koilocytes) were successfully genotyped for IL-1α intron 6 46bp VNTR.

The 9,9 genotype, associated with low secretion of IL-1α, was associated with LSIL (CIN I p=0.031, Koilocytes p=0.047 Fisher's exact test) but not with HSIL (p=0.082 Fisher's exact test) when compared to normal controls (Table 3.12).

*Table 3.12* IL-1α polymorphism in women with cervical disease

| Polymorphism | Cervical diagnosis | | | | |
|---|---|---|---|---|---|
| IL-1α (secretor phenotype) | Normal | Koilocytes | CIN I | CIN II/III | All CIN |
| 8,8 (high) | 3 (12%) | 2 (8%) | 0 | 2 (4%) | 2 (3%) |
| 8,9 | 12 (44%) | 3 (13%) | 3 (21%) | 15 (32%) | 18 (30%) |
| 9,9 (low) | 12 (44%) | 19 (79%) | 11 (79%) | 30 (64%) | 41 (67%) |
| Total | 27 | 24 | 14 | 47 | 61 |
| *p value* $\chi^2$ test | *reference group* | *0.047* | *0.031* | *0.082* | *0.028* |

With the number of patients studied there was no statistically significant association demonstrated between IL-1α genotype and either disease outcome at 12 months (p=0.409 Fisher's exact test) or high risk HPV infection (p=0.756 Fisher's exact test) (Table 3.13, Table 3.14).

*Table 3.13* IL-1α genotype and disease outcome at 12 months in patients with LSIL.

| | | IL-1α genotype | | | |
|---|---|---|---|---|---|
| | | 8,8 | 8,9 | 9,9 | Total |
| Disease outcome | Progression | 0 | 0 | 3 (10%) | 3 (8%) |
| | Persistence | 0 | 1 (16%) | 4 (13%) | 5 (13%) |
| | Regression | 1 (50%) | 4 (68%) | 18 (60%) | 23 (61%) |
| | Lost to follow up | 1 (50%) | 1 (16%) | 5 (17%) | 7 (18%) |
| | Total | 2 | 6 | 30 | 38 |

*Table 3.14* IL-1α genotype and high risk HPV infection

| High risk HPV infection | IL-1α genotype | | |
|---|---|---|---|
| | 8,8 | 8,9 | 9,9 |
| Positive | 2 (100%) | 3 (50%) | 19 (63%) |
| Negative | 0 | 2 (34%) | 7 (24%) |
| No result | 0 | 1 (16%) | 4 (13%) |
| Total | 2 | 6 | 30 |

### 3.4.5 IL-1Ra

Forty five normal controls, 67 patients with HSIL and 66 patients with histologically confirmed LSIL were successfully typed for IL-1Ra IVS2 86bp VNTR genotype. There was no statistically significant association demonstrated between IL-1Ra genotype and cervical disease (Table 3.15). Allele 2, which has been associated with epithelial disease was no more common in the cervical disease group than in normal controls (p=0.871 koilocytes, p=0.821 CIN I, p=0.996 HSIL Fisher's exact test).

*Table 3.15* IL-1Ra polymorphisms and cervical disease

| Genotype | Cervical diagnosis | | | | |
|---|---|---|---|---|---|
| | Normal | Koilocytes | CIN I | CIN II/III | All CIN |
| 1,1 | 22 (49%) | 24 (51%) | 9 (48%) | 27 (40%) | 36 (42%) |
| 1,2 | 17 (38%) | 18 (39%) | 8 (42%) | 33 (49%) | 41 (48%) |
| 1,3 | 0 | 1 (2%) | 1 (5%) | 0 | 1 (1%) |
| 1,4 | 3 (6.5%) | 1 (2%) | 0 | 3 (5%) | 3 (3%) |
| 2,2 | 3 (6.5%) | 2 (4%) | 0 | 4 (6%) | 4 (5%) |
| 2,4 | 0 | 1 (2%) | 1 (5%) | 0 | 1 (1%) |
| Total | 45 | 47 | 19 | 67 | 86 |
| Allele | | | | | |
| 1 | 42 (93%) | 44 (94%) | 18 (95%) | 63 (94%) | 81 (94%) |
| 2 | 20 (44%) | 21 (45%) | 9 (47%) | 37 (55%) | 46 (53%) |
| 3 | 0 | 1 (2%) | 1 (5%) | 0 | 1 (1%) |
| 4 | 3 (6.5%) | 2 (4%) | 1 (5%) | 3 (5%) | 4 (5%) |

There was also no association demonstrated between IL-1Ra genotype and disease outcome at 12 months (Table 3.16) or between IL-1Ra genotype and high risk HPV infection (Table 3.17).

*Table 3.16* IL-1Ra genotype and disease outcome

| Disease outcome | Genotype | | | | | | Allele | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1,1 | 1,2 | 1,3 | 1,4 | 2,2 | 2,4 | 1 | 2 | 3 | 4 |
| Progression | 2 | 5 | 0 | 0 | 0 | 0 | 7 (11%) | 5 (17%) | 0 | 0 |
| Persistence | 3 | 1 | 1 | 1 | 0 | 1 | 6 (10%) | 2 (6%) | 1 (50%) | 2 (67%) |
| Regression | 20 | 16 | 0 | 0 | 1 | 1 | 36 (58%) | 18 (60%) | 0 | 1 (33%) |
| Lost to follow up | 8 | 4 | 1 | 0 | 1 | 0 | 13 (21%) | 5 (17%) | 1 (50%) | 0 |
| Total | 33 | 26 | 2 | 1 | 2 | 2 | 62 | 30 | 2 | 3 |

*Table 3.17* IL-1Ra genotype and high risk HPV infection

| High risk HPV | Genotype | | | | | | Allele | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1,1 | 1,2 | 1,3 | 1,4 | 2,2 | 2,4 | 1 | 2 | 3 | 4 |
| Positive | 23 | 16 | 0 | 0 | 2 | 1 | 9 (28%) | 19 (63%) | 0 | 1 (33%) |
| Negative | 8 | 7 | 2 | 1 | 0 | 1 | 18 (56%) | 8 (27%) | 2 (100%) | 2 (67%) |
| No result | 2 | 3 | 0 | 0 | 0 | 0 | 5 (16%) | 3 (10%) | 0 | 0 |
| Total | 33 | 26 | 2 | 1 | 2 | 2 | 32 | 30 | 2 | 3 |

### 3.5 Extended haplotype analysis

Haplotypes were estimated for 27 normal controls, 49 patients with HSIL and 50 patients with histologically confirmed LSIL. These numbers represented the women who had been typed for both IL-1α and IL-1Ra (Table 3.18)

*Table 3.18* Incidence of each haplotype in normal controls, patients with HSIL and patients with LSIL.

| Haplotype | Cervical disease | | |
|---|---|---|---|
| | Normal | CIN I/Koilocytes | CIN II/III |
| 9,1 | 26 (48%) | 62 (62%) | 46 (47%) |
| 9,2 | 10 (19%) | 22 (22%) | 33 (34%) |
| 9,3 | 0 | 1 (1%) | 0 |
| 9,4 | 0 | 2 (2%) | 0 |
| 8,1 | 15 (28%) | 13 (13%) | 19 (19%) |
| 8,2 | 1 (2%) | 0 | 0 |
| 8,3 | 0 | 0 | 0 |
| 8,4 | 2 (3%) | 0 | 0 |
| Total | 54 | 100 | 98 |

There were no significant associations between any particular haplotype and cervical disease.

## DISCUSSION

### 4.1 Patient characteristics in this study

#### 4.1.1 Age

The ages of the patients with LSIL, HSIL and the controls were not significantly different. The peak age for CIN is 25-35 and our patients had a median age within this range (Brinton 1993). There were teenage patients included in the study who were less than the 1997 NHSCSP recommended age to commence screening of 20. In a study of teenage cytology, 1.6% (145/9094) were shown to have abnormal cytology (Macgregor 1978). The rates of disease outcome over 10 year follow up were similar to studies including a full age range (15% progression, 20% persistence and 65% regression) but no teenager progressed beyond CIN III before 21 years old. The Cancer Research Campaign (1994) reports the incidence of invasive cervical cancer in teenagers as two per million. In 2002 there were five deaths and 26 cases of cervical cancer in 15-24 year olds and 55,000 abnormal smears in 20-24 year olds (DOH 2003). These abnormal smears are therefore largely due to transient HPV infection and the majority resolve spontaneously over time. Collins et al established that HPVI is very common in the immediate period following first intercourse (Collins 2002). Wright et al showed that the risk period for cervical disease was 6-20 years after first intercourse (Wright 1984). These findings and those by Sasieni et al (Sasieni, Adams 2003) lead to the recommendation that routine screening need not begin until 25 years old (NHSCSP 2004). When opportunistic smears have been taken and prove to be abnormal in young women, their management should not differ significantly from that of women in their late 20s.

The criteria for a screening test were described by Wilson and Jungner in 1968 (Wilson 1968). These considerations have been updated by the UK National Screening Committee to include "the benefit from the screening programme should outweigh the physical and psychological harm" and "costs should be balanced in economic, psychological, social, and medical terms and with healthcare expenditures as whole" (UK National Screening Committee 2003). Given the low numbers of cases of cervical cancer in the under 25 year olds compared to the levels of abnormal smears the balance of

benefit does not outweigh the harm and the costs are not balanced. In addition Quinn et al called into question the effectiveness of screening women under 25 while demonstrating it's effect in other age groups (Quinn, Babb 1999). It should also be noted that screening is a method of identifying precancerous disease in asymptomatic women. Women under 25 with cervical disease may also present with symptoms. Abu, Jha and Rosenthal all examined women referred to colposcopy clinic with post coital bleeding and concluded that while cervical cancer is rare, CIN is more common in women with this presentation than in the general population and recommend that such women be seen in colposcopy clinic (Abu 2006, Jha 2002, Rosenthal 2001). This is a diagnostic rather than a screening approach which might offer a more targeted tactic for young women. A study of women under 25 who are now outside the cervical screening programme but who present with symptoms is indicated.

### 4.1.2 Parity

This study showed patients with increasingly severe disease were of increased parity. High parity is a risk factor for cervical disease (Schiffman 1993). High parity increases the risk of cervical cancer for HPV positive women (Munoz 2002). No pregnant patients were included in the study. Whilst their genotype would be unaffected, the evolution of disease process could be different from non pregnant patients because their cytokine profile differs as a consequence of pregnancy from non pregnant women with increased Th2 and reduced Th1 responses (Purtilo 1972). In addition, cervical HPV infection differs from non pregnant patients (Rando 1989, Schneider 1987).

### 4.1.3 Contraceptive use

The control patients were recruited from a family planning clinic and were therefore making active decisions about their fertility. They cannot be regarded as a matched control group in terms of contraception. Barrier contraception might protect the cervix against exposure to HPV. Initial studies linked the combined oral contraceptive pill to cervical cancer but when sexual behaviour is controlled for, the link is less clear. Moscicki showed that current use of the OCP is protective against HPV infection and is not a risk factor for LSIL (Moscicki 2001). Pooled data from previous studies suggests that short

term OCP use is not a risk factor for cervical cancer but use over 10 years may act as a cofactor for HPV and increase the risk of cervical cancer by up to four fold (Moreno 2002). In the study patients the COCP was the most popular form of contraception.

### 4.1.4 Smoking

Smoking is known to be a risk factor for cervical carcinoma (Winkelstein 1990). This link is independent of the association between smoking and sexual behaviour that has been thought to be a confounding factor. Nicotine and cotinine are found in cervical mucus at levels that correspond to cigarette consumption (McCann 1992). Smoking is known to suppress the immune response; smokers have lower numbers of Langerhans cells in the cervix than non smokers (Barton 1988, Poppe 1995). This immunosuppression may allow persistence of HPV infection thought to be necessary for disease progression. Smoking cessation has been shown to have a significant reduction in cervical lesion size in women with low grade cervical disease (Szarewski 1996). In our study, the control patients were significantly less likely to be smokers than the patients with low grade cervical disease and therefore cannot be seen as a matched control group with regards to smoking behaviour. We relied on self reported smoking behaviour. In Szarewski's study, self reported smoking behaviour was not confirmed by salivary cotinine tests in 8% of women. In our study there was no significant difference in self reported smoking between TNF$\alpha$ -308 genotypes.

## 4.2 Disease at recruitment

Power calculations indicated that 200 patients and 70 controls should be recruited in order to meet the calculated sample sizes. We saw all women referred to colposcopy clinic with smears showing borderline change or mild dyskaryosis in one inner city teaching hospital over a three year period. In all, 209 met the entry requirements and agreed to be recruited. Recruitment of suitable control patients was limited to 47. Ethical approval was granted for recruitment in the family planning clinic. However the approval was limited to only the researcher recruiting and approval was not given for the researcher to approach patients directly to ask them to participate. Written information

was provided in the waiting room and interested women with a complete and negative smear history who were prepared to undergo venepuncture were asked to make themselves know the researcher. In addition, at the time of the study, there was extensive publicity relating to the care of children receiving complex cardiac surgery in Bristol and this may have affected the willingness of Bristolians to participate in medical studies (Bristol Royal Infirmary Inquiry 2001). Thus the study may be under-powered to identify a difference between controls and patients for all polymorphisms but TNFα -308.

The diagnosis at recruitment was made using a combination of cytology, colposcopy and histology of a directed punch biopsy specimen. The use of punch biopsy in any prospective study is controversial given the possibility of altering the natural history of disease by this intervention. However it improves diagnostic accuracy and it is established clinical practice as part of diagnosis of low grade cervical disease.

Of the 209 patients recruited with low grade smears, 22% were deemed to have a normal cervix on reaching the colposcopy clinic. They represent a group of women with regressing lesions whose cytological abnormality has resolved. The nature of the screening programme is such that many women will develop and resolve minor cytological abnormalities in the interval between smear tests. These will never be detected.

Sixteen per cent of women with low grade smears had HSIL at recruitment. This is in keeping with previous studies (Jones, Jenkins 1992, Shafi, Luesley 1997). These women represent a group whose disease may be underreported by cytology. They may also include women whose cervical disease progresses between the time of smear taking and colposcopy clinic, typically at least 12 weeks. It would not be ethical to withhold treatment in these women to establish whether their disease continues to progress. Two women had a LLETZ showing CIN I when their original punch biopsy showed CIN II. These women appear to have had very small lesions that were completely excised by punch biopsy and this may explain why their original cytology was low grade. Buxton noted that in small lesions punch biopsy is more likely to give a more severe diagnosis than excisional biopsy (Buxton, Luesley 1991).

When these two groups of women were excluded from the study only 129 (62%) of the recruited patients had low grade cervical disease. Twenty three patients (11% of those recruited) were shown to have CIN I. This demonstrates that CIN I is a rare condition to diagnose. However in only 40% of patients with low grade disease, the cytology and histology agreed. A single pathologist examined the smears and biopsies to reduce inter-observer variability. Previous studies suggest concordance between cytology and histology is better in high grade disease and cervical cancer that in low grade disease (Tabbara 1992). CIN I may be more common than our study suggests but resolve or progress relatively quickly.

### 4.3 Disease outcome

One hundred and two patients were followed up for 12 months. Of the remainder, two patients requested treatment and were withdrawn from the study. A further 25 (19%) were lost to follow up. These women were sent three appointments by letter and did not attend or rearrange their appointments. This level is higher than the NHSCSP accepted rate of 15% for non-attendance at colposcopy clinic. The NHSCSP rate is the recommendation for new and follow up patients and those with all grades of CIN. The reasons for defaulting to follow up are varied. Some women find the colposcopic examination painful or embarrassing and are unwilling to repeat it. Others move house or change name and do not receive their appointment. The challenge for colposcopists seeing women with low grade abnormalities is to offer enough reassurance that immediate or radical treatment is not required while ensuring that the importance of ongoing follow up is understood. Women prefer an active management strategy in management of mildly abnormal smears although colposcopy increases anxiety levels over cytological surveillance (Jones 1996, Meana 1999). A high rate of default has been seen in previous studies of the management of low grade disease and is part of the argument for immediate colposcopy rather than cytological surveillance (Flannelly, Anderson 1994).

Twelve month follow up is relatively short in terms of the natural history of cervical disease. Our data shows that by this time 64% of patients had either progressed to HSIL and treatment or regressed to discharge from colposcopy

clinic and to cytological surveillance. A further 21% had stopped follow up by defaulting or requesting treatment, leaving only 15% of patients with LSIL (or 9% of those originally recruited) with ongoing LSIL at this time. This shows that the majority of low grade disease will progress or regress relatively quickly but that for the remainder, prolonged colposcopy clinic follow up results in a high rate of default and ongoing patient anxiety.

Nine of the eleven patients with disease progression (82%) were found to have high grade disease at six months. Given the diagnostic uncertainty previously discussed, it is possible that these women had HSIL at recruitment that was not apparent. Cervical screening and colposcopy assess the cervix at a single point in a dynamic disease process. The women whose disease progressed more quickly may have been at a more advanced point in the disease process than those who did not progress during the study period. Although all the women recruited had persistent disease for at least 12 months before recruitment, some of these women will have had disease for up to five years since their previous normal smear. Others will have developed disease more recently. Severity of disease is affected by viral load. Although we didn't assess viral load in this study it is possible that women experiencing more rapid disease progression had increased levels of HPV.

The rate of progression was 8% overall, but 30% in women with CIN I and only 4% in those with borderline nuclear change or koilocytosis. The expected rate varies between previous studies depending on recruitment criteria and length of follow up but is generally thought to be 10% for LSIL. This supports the idea that borderline nuclear change on cytology is simply a manifestation of HPV infection and has much lower progressive disease potential than actual dysplasia. However disease regression was the most common outcome, 45% of women with CIN I or mild dyskaryosis and 65% of women with borderline nuclear change or koilocytosis. Further analysis identified one woman with koilocytic change and normal cytology at her first visit, who had CIN I and normal cytology at six months. She was classed as persistent disease as she continued to have LSIL but could be regarded as progressing from koilocytosis to CIN I. At 12 months she had koilocytic change and normal cytology once again. She was negative for high risk HPV throughout.

The management of women with smears suggesting low grade cervical disease remains controversial. Although the majority will regress spontaneously a significant number of women will have high grade CIN. Immediate referral to colposcopy allows more accurate diagnosis via colposcopic examination and punch biopsy and represents the active management preferred by women. However such women should not be treated without histological confirmation of high grade disease especially since increasing evidence suggests poorer reproductive outcomes in women following such treatment (Sadler 2004). This study agrees with previous work that differentiates between borderline smears and those showing mild dyskaryosis. Mild dyskaryosis and CIN I represent more significant disease than borderline change and koilocytosis and therefore cytological surveillance remains appropriate for women with borderline smears, especially those in younger age groups. HPV testing may prove a useful adjunct to the management of these women and the TOMBOLA trial is assessing its' role (Cotton 2006).

Smoking was an important factor in disease regression with 42% of smokers and 67% of non smokers undergoing disease regression. This supports the previous studies implicating smoking as a cofactor in cervical disease as discussed above.

### 4.4 HPV infection.

Overall 62% (128/209) of the patients recruited with low grade smears were positive for one or more high risk HPV types. Those patients with a normal cervix were significantly more likely to be HPV negative than those with HSIL ($p=0.000005$). This difference would be expected given the known role of HPV in cervical disease. However 39% of women with a normal cervix were positive for high risk HPV. It is estimated that only 3 in 10,000 women infected with high risk HPV will develop cervical cancer. There has been considerable interest in the role of HPV triage in women with low grade cervical disease or low grade cytology to identify those most at risk of progression. The NHS Health Technology Assessment Programme reviewed the evidence in 1999 and found potential roles for HPV testing in the NHSCSP in three areas. These were the primary screening of women over 35 years in whom the false

positive rate is lowest, the follow up of women treated for HPV positive cervical disease to detect early recurrence and the improvement of management of women with low grade or borderline smears (Cuzick, Sasieni 1999). Subsequently Rebello et al showed that HPV testing with Hybrid Capture II was 93% sensitive and 55% specific (1pg/ml cut off) for identifying HSIL in women with persistent borderline or low grade cytology. They showed greater specificity and sensitivity for women over 30 and for those with borderline cytology compared to younger women and those with mild dyskaryosis (Rebello, Hallam 2001). Manos investigated women with a single ASCUS (equates to borderline) smear and found HPV testing with Hybrid Capture II to be 89% sensitive and 64% specific for HSIL (Manos, Kinney 1999). The ALTS group found high levels (over 80%) positivity for high risk HPV in women with LSIL and concluded that the role of HPV in triage is limited by these high levels of infection (ALTS 2000). These studies differ in their conclusions largely because of their differing populations. The recruitment of women following a single abnormal smear test or after persistent abnormalities alters the results given the high expected level of regression in the former group. These studies also recruited women with either borderline or mild dyskaryosis on cytology. Our study demonstrated significantly different levels of high risk HPV infection in women with borderline smears or koilocytic change on histology (56%) compared to those with mild dyskaryosis or CIN I (72%) ($p=0.05$). Denise Zielinski et al demonstrated that regression of cervical disease in women with low grade smears is strongly associated with viral clearance and that viral clearance precedes normal cytology by an average of four months (Denise Zielinski, Snijders 2001).

Multiple HPV infection with two or more infecting types was common in this study at 46% of those high risk HPV positive. Rousseau et al found the risk factors for multiple HPV infection were the same as those for single HPV infection (Rousseau 2003). They also showed the rate of multiple infection in their population to be 20% but showed no difference in levels of persistent infection between single and multiple infections (Rousseau 2001). Nuovo et al examined HPV infection in women with CIN and showed 2.4% (5/204) multiple infection when examined using ISH but 18% (6/33) when examined

using PCR. They suggest infection with one type of HPV may inhibit replication by any subsequently infecting types (Nuovo 1991). More recent work from Edinburgh shows similar high rates of multiple HPV infection to this Bristol study (Cuschieri 2004).

There is considerable geographical variation between different HPV types (Bosch, Manos 1995). In women with cervical cancer the most common infecting HPV type is HPV 16 (51.5%) with 79.8% of cervical cancer patients positive for HPV 16, 18, 31 or 45. Sixty five per cent of patient in our study recruited with low grade smears were positive for these types.

The majority of patients in our study who experienced disease progression were positive for high risk HPV (9/11 82%). This was significant (p=0.05) and supports the theory that HPVI is required for disease progression. The single woman with CIN II at six months who was high risk HPV negative is an exception. It is possible she had previously been infected with high risk HPV but had cleared the virus prior to her first visit. Virus clearance usually occurs prior to cytological regression. Had it been ethical to follow this woman further without treatment, it is possible that her cervical disease would also have regressed. However our practice was to treat women with CIN II or greater. It is also possible that she had CIN II at recruitment and, therefore did not progress within the duration of the study.

The majority of patients who experienced disease regression were also positive for high risk HPV and patients positive for HPV were not significantly more likely to progress than those negative for HPV. This highlights the possible drawbacks of HPV testing as a triage for women with low grade cytology. While a negative HR HPV test may provide reassurance, a positive test should not be taken as a marker for disease progression.

The patients who experienced disease progression were infected with a variety of HPV types; 16, 18, 31, 33, 39, 51, 52, 66 and 73. Much work has focused on HPV 16 and 18 as the most important HPV types in cervical carcinoma. In our study only 54% of the patients whose disease progressed and who underwent treatment were infected with these types. In our population using HPV types 16 and 18 as a triage for progression would have missed 36% (4/11) of those who progressed.

Low risk HPV types were most commonly seen as part of multiple infection. They are not associated with carcinogenesis but they may contribute to abnormal smears by producing koilocytic change.

Previous investigators have reviewed characteristics of HPV in an attempt to predict which HPVI will lead to serious cervical disease. Within the L1 region polymorphisms have been identified, the rate of nucleotide diversity being between 0.2-2.9% (Stewart 1996). The E7 region was examined in Korean women with cervical disease. There is a range of variants with the most common variant in Korea affecting the amino acid related to high antigenicity and binding to retinoblastoma protein. This variant is more common in women with cervical carcinoma suggesting a more profound oncogenic potential (Song 1997). A study in Sweden examining E6 and E7 variants examined women with cervical cancer and CIN III(Zehbe 1998). Those women with cervical cancer were almost exclusively positive for HPV16 E6 variants (94%) and rarely harboured the prototype (6%), whereas CIN III demonstrated a more uniform distribution of variants (56%) and prototype (44%; P = 0.013). All variants in this study contained variations that were identified in areas likely to be important for protein-protein interaction with p53 or in areas of immunological significance. The most frequent E6 variation was seen at residue 83. E7 variations were extremely rare in this group and were only detected together with E6 variations in 4% of CIN III and in 6% of cervical cancer cases, suggesting that the HPV16 E7 but not the HPV16 E6 oncoprotein is highly conserved in vivo. This indicates that HPV16 E6 variants, specifically those containing the substitution at residue 83, may be more oncogenic than the prototype and thus carry a higher risk for the development of invasive cervical disease. This may be due to subtle differences in the type of transformation produced or to evasion of host immune defences. However by contrast a German study of variants of E6 and E7 found no difference in distribution of variants between women with normal cervices, CIN of all grades or cervical cancer (Nindl 1999). Van Duin et al examined HPV variants in combination with p53 codon genotypes and found that in women with p53 Arg/Arg genotypes, infection with a particular HPV E6 variant was more common in those with cervical cancer than those with normal histology (van Duin 2000). This study suggests a combination of HPV

variants and host characteristics may be important. This theory is further supported by the association of particular HLA class II types with cervical cancer. Certain HLA class II haplotypes (such as DRB1*1501-DQB1*0602) were associated significantly, while DR13 haplotypes were negatively associated with cervical carcinoma (Apple 1994).

### 4.5 Cytokine gene polymorphisms

When examining cytokine gene polymorphisms we included only women whose histology confirmed LSIL. This was in order to optimise diagnostic accuracy as much as possible given the difficulties in diagnosis previously discussed.

While it is clear HPVI is vital in the development of cervical disease, host factors are likely to determine which infections lead to cervical cancer and which to a transient mild cytological abnormality, if any abnormality at all. TNFα is a potent pro-inflammatory cytokine where the polymorphisms in the promoter region have been shown to have a 6-9 fold effect on the level of transcription (Wilson, Symons 1997). The GG genotype of TNFα -308 (corresponding to a low secretor phenotype) was associated with CIN I, II and III ($p=0.01$) with the association being strongest with CIN I ($p=0.004$). TNFα levels have been shown to be reduced in cervical cancer by previous studies and these findings support a hypothesis that cervical disease at all grades is influenced by levels of this cytokine (De Jaco, Asselain 1991, Woodworth, McMullin 1995). However no significant association was shown between this polymorphism and koilocytic change suggesting that koilocytic change represents the presence of HPVI rather than clinically important cervical disease. No association was demonstrated between CIN and TNFα -238 polymorphism. The study is underpowered to detect a difference related to this polymorphism but the numbers in each group are similar and it is unlikely any association exists. No statistically significant association was shown between polymorphisms of TNFα and disease outcome or high risk HPVI in women with LSIL. High levels of TNFα have been shown to reduce the expression of HPV 16 (Kyo, Inoue 1994). In addition, cervical cell lines immortalised by HPV 16 and 18 do not respond to TNFα as well (Woodworth,

McMullin 1995). In this study, although HPV 16 and 18 were the most common infecting types they were still present in a minority of patients. HPV 16 and 18 are associated with cervical cancer and the presence of a wide range of other HPV types reflects the population with low-grade cervical abnormalities.

The polymorphism in the promoter region of the IL-1α has been identified but not shown to have a statistically significant influence on levels of cytokine production. There does appear to be a trend in this relationship. In this study, the 9,9 genotype, tending to low secretion of IL-1α, is associated with koilocytic change and CIN I but not with HSIL (p=0.031, 0.047 and 0.082). While this finding cannot be as certainly explained by cytokine levels as that associated with TNFα it appears that the polymorphism does exert an effect. An earlier study showed a strong association between IL-1β and cervical disease (Majeed, Glew 1999). The close geographical links between IL-1α and IL-1β and their homology of structure supports the data for such a link. IL-1Ra is also closely associated with both cytokines and acts as a regulator for their release. The rare allele 2 is linked to high secretion of IL-1Ra and thus low secretion of IL-1α. In this study we were unable to demonstrate a link between allele 2 and cervical disease of any grade. It is possible that the polymorphism of IL-1α promoter is linked with LSIL not simply by levels of cytokine production.

Cytokine gene polymorphisms and levels of cytokines in cervical disease are important in relation to HSIL and cervical cancer in terms of therapeutic strategies. In LSIL, prognosis is important to allow prompt treatment to those women who will progress and reassurance for the majority of women, not at risk of serious cervical disease. One of the aims of this study was to identify prognostic links between cytokine gene polymorphisms and HPVI and with cervical disease outcome. No associations were demonstrated between any of the three polymorphisms examined and HPVI or cervical disease outcome suggesting that these polymorphisms alone are not prognostic markers. Because of the complex interactions of the cytokine network, the hypothesis that a combination of cytokine polymorphisms may be more important than single polymorphisms arises. The statistical method for haplotype

reconstruction we used showed no associations between LSIL and any particular haplotype cytokines. Further investigation of combinations of cytokine polymorphisms may reveal further associations with cervical disease.

### 4.6 Strengths and limitations of the study

This study sought to address an important clinical problem; the management of women with smears suggesting low grade cervical disease, by identifying a genetic marker for disease risk rather than relying on morphological diagnosis. By assessing the individual's potential to mount an effective immune response to viral infection it avoided the effect of some confounding factors such as inter-current infection on directly measured cytokine levels.
The study was underpowered in control numbers due to the ethical constraints placed on recruitment of control patients. In addition despite recruiting the desired number of patients, the diagnosis of CIN I proved to be rarer than expected. Since all patients with low grade smear abnormalities in the study hospital were seen by the research team, the only way to recruit more patients with CIN I would have been to include more than one centre.
The cytokine gene polymorphism analyses proved less successful than in previous studies with a greater than expected five per cent drop out rate despite concerted attempts to obtain a complete results set. These unsuccessful tests may have biased the results as the reasons for lack of success were unclear and may have been associated with a consistent factor. In the same way exclusion of patients lost to follow up from the statistical analysis may have biased the results.
The disease outcomes may lack accuracy as patients with normal cervix at colposcopy and cytology were not assessed histologically because it was deemed not to be ethical to take a cervical biopsy if not clinically indicated.

### 4.7 Implications of the research

It is increasingly recognised that cytokines have a significant impact on susceptibility to and outcome of a range of diseases. Because of the highly complex network in which they operate and because of the effects of confounding factors such as smoking, it may not be possible to demonstrate an effect of a single cytokine gene polymorphism. However associations

between cervical disease and polymorphisms in the promoter genes for two cytokines (IL-1α and TNFα) have been identified in this study. Since this study was undertaken, vaccines against HPVI have been developed and licensed, underlining the importance of the immune response to HPVI in the prevention of cervical cancer. The host's innate ability to mount an immune response in response to vaccination or infection may affect the success of these welcome developments.

### 4.8 Future areas of work

The long term clinical follow up of those women whose disease regressed would demonstrate the risk of recurrence of CIN in women with regressing low grade cervical abnormalities.

IFN-γ has antiviral activity both by inhibiting HPV 16 gene transcriptions and by inducing cytotoxic T cells. The gene for this cytokine shows a variable length CA repeat in the first intron of the gene. The presence of allele 2 is associated with a high secretor phenotype for IFN-γ (Pravica 1999).

IL-18 is linked to IFN-γ production. There are single gene polymorphisms in the gene promoter region at -105 A/C and -137 G/C which are linked to expression levels (Arimitsu 2006).

Assessment of these further cytokine gene polymorphisms and combination with those already studied in this thesis and might identify a high risk haplotype for disease progression.

Such a haplotype might form a useful part of a predictive model for cervical disease progression in combination with HPVI and smoking.

**Achievement of the aims of this thesis**

To determine the incidence and type of HPV infection in women presenting to colposcopy clinic with smears suggestive of low-grade cervical disease.

The incidence of high risk HPVI in women presenting to colposcopy clinic with smears suggestive of low grade cervical disease was high (62% of women recruited). Multiple infecting types were common (46%) and HPV 16 and 18 were the most common types identified. However high risk HPVI was not significantly associated with disease progression.

To compare the cytokine gene polymorphisms found in IL-1α, IL-1Ra and TNFα in women with normal cervical cytology and with histological low-grade and high-grade cervical disease.

Polymorphisms in the promoter regions of IL-1α and TNFα were associated with cervical disease compared to normal controls. Alleles representing low secretor phenotypes were more common in women with LSIL. This correlates with the previously reported association between a reduction in Th1 cytokines and cervical disease. No association was demonstrated for the polymorphism of IL-1Ra. These polymorphisms were not shown to be linked to high risk HPVI. However the study was underpowered to detect associations with these polymorphisms and the lack of association may reflect a lack of patient numbers.

To examine the disease outcome in women with low-grade cervical disease in relation to HPVI and cytokine gene polymorphism.

The disease outcome for women with smears suggesting low grade cervical disease was not shown to be linked to high risk HPVI or the cytokine gene polymorphisms examined.

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**Appendix 1 - Technique of colposcopy**

Having obtained verbal consent, the woman is placed in modified lithotomy and the cervix is exposed using a warmed bivalve speculum. The vulva, vagina and cervix are visually inspected. Colposcopy is deemed to be satisfactory if the transformation zone is entirely visible. A cervical smear is taken at this point if indicated. Excess mucus is removed from the cervix with a saline soaked cotton wool swab. 3% Acetic acid is applied to the cervix with cotton wool and left for 60 seconds. Acetic acid causes coagulation of the nuclear protein. Normal cells still appear pink as the amount of nuclear protein is such that the vessels in the underlying stroma remain visible. Abnormal cells have a higher nuclear cytoplasm ratio and a higher nuclear protein density; this undergoes more coagulation and the cells appear white as the underlying vessels are obscured. The more abnormal the cell, the higher the concentration of protein and the whiter the cells appear. This effect is reversible and repeat applications of acetic acid allow a more prolonged inspection.

Lugol's iodine is then applied to the cervix with cotton wool. The iodine stains glycogen, which is abundant in mature squamous epithelium but absent in abnormal cells. The abnormal cells appear pale yellow while the normal stain deep brown.

For diagnostic purposes a colposcopic directed punch biopsy of the most abnormal areas may be taken. Haemostasis is achieved following biopsy either with silver nitrate sticks or with Monsel's solution (ferric subsulphate) on a cotton bud.

Where treatment is required the commonest method is the LLETZ (Large Loop Excision of the Transformation Zone). The cervix is anaesthetised with local anaesthetic plus a vasoconstrictor injected at four points. The patient has a diathermy earth plate applied to her thigh and a smoke extractor is attached to the speculum. A diathermy loop appropriate to the size of the lesion is placed on the cervix just lateral or superior to the area to be removed. The loop is passed smoothly through the tissue in cut mode. The specimen is removed intact and the resulting crater is coagulated with ball diathermy and if necessary Monsel's solution. The wound is left open. This is shown to be a safe and effective treatment (Luesley 1990).

## Appendix 2

**PATIENT INFORMATION SHEET**

We are undertaking some work on how the immune system operates in the cervix in various stages of health and disease. We would welcome your help with our study. Althougth this research will not affect the advice or treatment given to you, it is hoped that in future we will have a better understanding of how to effectively treat women with cervical smear abnormalities.

If you are willing, we would like to take a simple blood test from you. We would also like to take a small biopsy from the cervix during the course of your treatment in the colposcopy clinic, or (if you are going to theatre) during the course of your operation. You are under no obligation to participate by giving a blood test and biopsy, and you are free to change your mind about helping our research at anytime without needing to give a reason.

If you would like more information then please do not hesitate to ask the doctor who has shown you this form. If you have any queries at any time then please phone Miss Susan Glew on 9285290

SUSAN GLEW
November 1996

U B H T
TEACHING CARE

The United Bristol Healthcare NHS Trust

**PATIENT INFORMATION SHEET:**
**WOMEN WITH NORMAL CERVICAL SMEARS**
**STUDY OF PROGNOSTIC FACTORS IN CERVICAL DYSPLASIA**
**(predicting which women with mildly abnormal cervical smears will need treatment)**

## WHAT IS THE STUDY ABOUT?

We are researching how the immune system works in the cervix in health and disease. Our goal is to develop a blood test that will predict whether women with minor cervical smear abnormalities will need treatment, or whether the abnormality will clear up on its own. To do this we need to study the blood of women who have had cervical smear abnormalities, and then see if it differs from that of women with normal smears. We will be looking at the genes that control the immune system, which vary from woman to woman.

## WHY HAVE I BEEN APPROACHED, AND WHAT IS INVOLVED?

To complete our research we require blood specimens from women volunteers who have always had normal cervical smears. If you fall into this group and are agreeable we would like to take a simple blood test. We will also need to ask your age, when your last smear was (approximately) and whether or not you have had any pregnancies. Any information you give will be stored anonymously using a code number. If you agree to take part there will be no benefit to yourself. Your generosity will help our understanding of why cervical smear abnormalities arise in some women but not in others. There are no major risks to giving a blood test but you may experience a small amount of discomfort at the time, and there is a small chance you may have a bruise on your arm afterwards.

## IS PARTICIPATION OPTIONAL?

Absolutely. You are under no obligation to give a blood sample, and you are free to change your mind at any time without needing to give a reason. Whether or not you give a blood test, your treatment in the clinic will be exactly the same.

## WHAT IF I HAVE SOME QUESTIONS?

If you would like more information then please do not hesitate to ask the doctor who has shown you this form. If you have queries at a later time then please telephone Miss Susan Glew or Dr Alison Kirkpatrick on 0117 928 5291.

Whether or not you are able to help with the study we would like to thank you for taking the time to read this information sheet.

POSITIVE ABOUT DISABLED PEOPLE

The United Bristol Healthcare NHS Trust
Tel 0117 923 0000 Minicom 0117 934 9869

**Study Title: Prognostic Factors in Early Cervical Dysplasia**

*Please ask the patient to complete the following:*

| | *Please cross out as necessary* |
|---|---|
| Have you read the Patient Information Sheet? | Yes / No |
| Have you had an opportunity to ask questions and discuss this study? | Yes / No |
| Have you received satisfactory answers to all your questions? | Yes / No |
| Have you received enough information about the study? | Yes / No |

Who have you spoken to? .....

Do you understand that you are free to withdraw from the study:

- At any time
- Without having to give a reason for withdrawing
- And without affecting your future medical care — Yes / No

Do you agree to take part in this study? Yes / No

Signed..... Date.....

(Name in block letters) .....

Signed (Researchers):..... Date.....